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Evidence for Boundary Lipid in Membranes

(membranous cytochrome oxidase/spin label/electron spin resonance)

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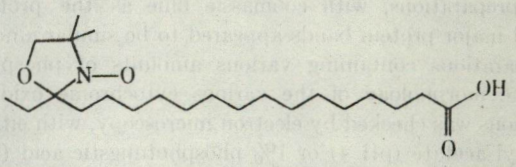
ABSTRACT **Cytochrome oxidase (EC 1.9.3.1) isolated from beef-heart mitochondria with an appropriate phospholipid content forms vesicular structures. Lipid–protein interactions in this model membrane system were studied with the lipid spin label, 16-doxylstearic acid. As the phospholipid/protein ratio is varied, two spectral components are observed. At low phospholipid/protein ratios (≤0.19 mg of phospholipid per mg of protein) the lipid spin label is highly immobilized. At higher phospholipid content an additional component characteristic of fluid lipid bilayers is evident. By summation of digitalized spectra and subsequent integration it was shown that all composite spectra could be approximated by assuming only two components are present, and that the amount of phospholipid bound to the protein is independent of the extent of the fluid bilayer region. The experimentally determined amount of phospholipid for maximum occupancy of protein-bound sites is about 0.2 mg of phospholipid per 1.0 mg of protein. Calculations show that this ratio is consistent with a single layer of phospholipid surrounding the protein complex. The data are interpreted as evidence for a boundary of immobilized lipid between the hydrophobic protein and adjacent fluid bilayer regions in this membrane model system.**

There is compelling evidence for the existence of phospholipid bilayers in biological membranes. The results of x-ray diffraction studies (1), differential thermal analysis (2), and spin labeling (3) have all indicated a similarity in lipid behavior between phospholipid bilayers and membranes. Evidence is also accumulating for the existence of globular amphipathic membrane proteins extending into or through the lipid regions of the membrane (4). Assuming this general model to be the case, there must exist a boundary between the fluid bilayer region and the membrane proteins. An interesting question arises as to the properties of the lipid–protein interface. Initially, a well-defined membranous preparation with relatively high protein content and easily characterized functional properties is a good system in which to examine lipid–protein interactions. For this study, we chose cytochrome oxidase (EC 1.9.3.1), which forms a model membrane system with partially-characterized components and functional properties (5, 6). At phospholipid/protein ratios of 0.3–0.7 (w/w), cytochrome oxidase, prepared by the general method of Sun *et al.* (7), spontaneously forms membranous vesicles (Fig. 1). These vesicular structures are essentially an artificial membrane system, but it is reasonable to suppose that interactions between the cytochrome oxidase protein complex and the phospholipids are meaningful in relation to similar associations in the intact inner mitochondrial membrane. In this paper we report a study of membranous cytochrome oxidase of various phospholipid contents that contains a small concentration of the spin label, 16-doxylstearic acid (8).



The properties of the protein–lipid boundary and the bilayer region are examined by electron spin resonance (ESR) spectroscopy.

Abbreviations: ESR, electron spin resonance; 16-doxylstearic acid, the 4′,4′-dimethyloxazoladine-*N*-oxyl derivative of 16-ketostearic acid.

MATERIALS AND METHODS

Preparation of Cytochrome Oxidase. Beef-heart mitochondria were prepared by the method of Crane *et al.* (9), except that 10 mM Tris·HCl (pH 7.8) replaced the phosphate buffer. These mitochondria were used to prepare membranous cytochrome oxidase by the general method of Sun *et al.* (7). Briefly, mitochondrial paste was suspended at 30 mg/ml in 0.25 M sucrose–10 mM Tris·HCl (pH 7.1), containing 1 mM EDTA, Triton X-114 added to 0.6 mg of Triton per mg of protein, and solid KCl added to a final concentration of 0.2 M KCl. After it was stirred for 30 min on ice, the suspension was centrifuged three times (78,000 × *g* for 60 min); each time the supernatant and pellet were discarded and the fluffy middle layer was retained. After the final centrifugation, protein concentration was adjusted to 20 mg/ml. The mixture was treated with Triton X-100 (1 mg/mg of protein) and solid KCl to 1 M. It was stirred on ice for 30 min and centrifuged at 105,000 × *g* for 30 min. The pellet was washed twice by centrifugation or by overnight dialysis against Tris·HCl (pH 7.1). The cytochrome oxidase preparation was stored at a concentration of 20 mg/ml at −20°.

These membranous cytochrome oxidase preparations varied in phospholipid content from 0.33–0.49 mg of phospholipid per mg of protein, assuming an average phospholipid molecular weight of 775. Additional lipid was incorporated by the method of Fleischer and Fleischer (10); unincorporated lipid was removed by sucrose density centrifugation. The maximum phospholipid/protein ratio attained was 0.73, which corresponds closely to the value of 0.70 calculated from the data of Chuang *et al.* (11) as the maximum phospholipid incorporation they could obtain.

Phospholipid content was reduced by successive 30-min extractions with cold 95% aqueous acetone (10) followed by

centrifugation, rotary evaporation, and suspension in buffer. Cytochrome oxidase lipids consisted of the pooled supernatants from the successive acetone extractions. Lipids extracted in this fashion contain an estimated 5-7% protein.

In the manner described, a series of cytochrome oxidase samples were prepared that ranged from 0.10 mg of phospholipid per mg of protein to 0.73 mg of phospholipid per mg of protein. All samples had a heme concentration of 7.8–8.5 nmol of heme *a* per mg of protein and had the characteristic optical spectrum of native cytochrome oxidase. All were active, as estimated by the method of Smith (12).

Phosphorous was measured by the method of Chen *et al.* (13), protein was determined by the method of Lowry *et al.* (14), heme *a* was estimated by the method of Williams (15), and cytochrome oxidase activity was determined by the method of Smith (12). Electrophoresis on 5% acrylamide gels containing 1% sodium dodecyl sulfate and 5 mM 2-mercaptoethanol (16) was used to characterize the cytochrome oxidase preparations, with coomassie blue as the protein stain. All major protein bands appeared to be similar among the preparations containing various amounts of phospholipid. The morphology of the various cytochrome oxidase preparations was checked by electron microscopy, with either 1% uranyl acetate (pH 4) or 1% phosphotungstic acid (pH 5) as negative stains. At ratios of 0.24 mg of phospholipid per mg of protein or less, cytochrome oxidase appeared amorphous or sheet-like by electron microscopy; at ratios of 0.33 or higher, the cytochrome oxidase was vesicular, similar to earlier observations (11).

Preparations of Spin-Labeled Cytochrome Oxidase. Cytochrome oxidase suspensions were washed twice by centrifugation and suspended in 10 mM phosphate buffer (pH 7.0). The spin label, 16-doxylstearic acid (Syva Associates), in chloroform solution was evaporated on the bottom of a vial, which was then aspirated to remove traces of chloroform, the buffered cytochrome oxidase suspension was added to the vial containing the thin film of spin label, and the suspension was thoroughly mixed by brief low-power sonication (Heat Systems Ultrasonics, Inc. bath sonifier, 40 W, 2 min) and then allowed to stand on ice for 30 min before a sample was withdrawn for ESR spectroscopy. Labeling was kept constant at 25 nmol of spin label per mg of protein. No spin-spin interactions were seen at double this concentration, even in the samples containing the lowest lipid concentration.

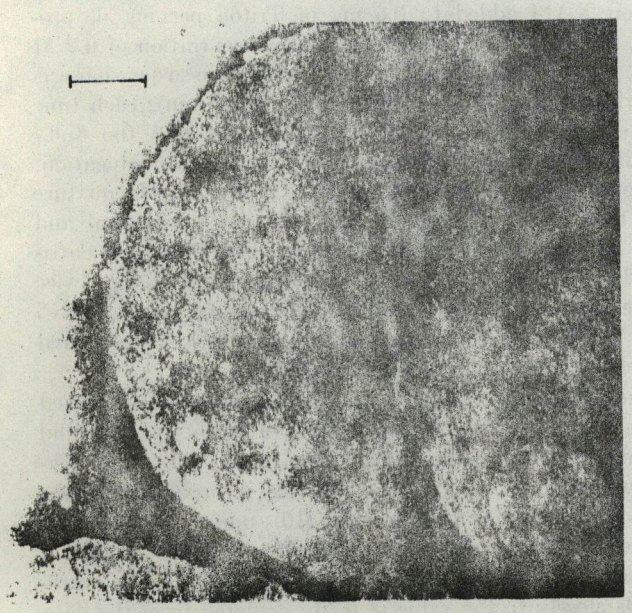


FIG. 1. Electron micrograph of membranous cytochrome oxidase (mg of phospholipid per mg of protein ratio is 0.49), negatively stained with 1% phosphotungstic acid (pH 5) showing the vesicular structure. Micrograph taken by Mr. William Colquhoun. (*Bar* = 0.1 μm).

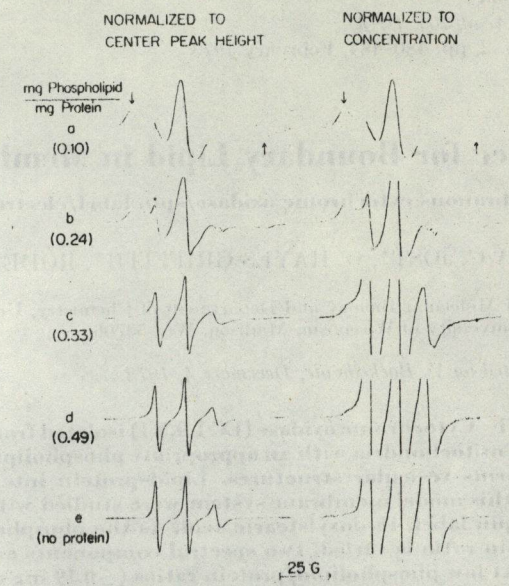


FIG. 2. ESR spectra of 16-doxylstearic acid spin label in buffered aqueous dispersions of membranous cytochrome oxidase with various lipid contents. The lipid to protein ratio expressed as mg of lipid per mg of protein is indicated at the far left. *Left*, spectra normalized to the center-line height; *right*, the same spectra normalized to give equivalent values after two integrations, i.e., the right column represents constant concentration.

ESR Measurements. All ESR measurements were made on a Varian E-3 spectrometer interfaced with an 8K Varian 620/i digital computer, with the scan speed controlled by an external oscillator. The first-derivative spectra were digitalized and stored on paper tape for later replotting, integration, subtraction, or addition (17). The usual spectrometer settings were microwave power 5 mW, modulation amplitude 1 G, scan range 100 G. The filter time constant varied from 0.3 to 1 sec for scan speeds of 10–30 min, depending on the line width associated with the sample. All spectra were recorded at room temperature unless otherwise noted.

RESULTS AND DISCUSSION

Spin-labeled cytochrome oxidase

ESR spectra were recorded for preparations of spin-labeled cytochrome oxidase containing increasing concentrations of lipid. The amount of lipid in each of the samples is expressed as the ratio of mg of phospholipid per mg of protein; the samples contained lipid/protein ratios of 0.10, 0.15, 0.19, 0.24, 0.25, 0.33, 0.39, 0.43, 0.49, and 0.73. In addition, a sample of lipids extracted from membranous cytochrome oxidase served as a reference sample.

The spectra of representative samples are shown in Fig. 2, arranged in order of increasing phospholipid content. The

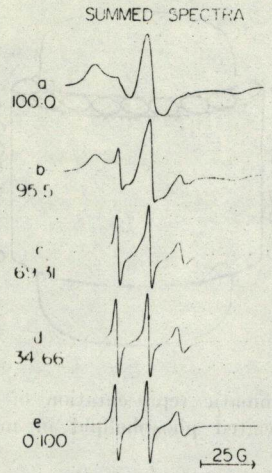


FIG. 3. Synthesized spectra obtained by summation of various amounts of spectrum *a* (lipid/protein ratio of 0.10) and spectrum *e* (lipids extracted from membranous cytochrome oxidase) of Fig. 2. The ratios on the *left* are the fraction of the total absorption contributed by each spectrum (*a*:*e*). The summed spectra have then been normalized to the same center-line height. Note the general similarity of the synthesized spectra *b*, *c*, and *d* to the corresponding experimental spectra (Fig. 2, *b*, *c*, and *d*).

spectral data are scaled in two different ways to illustrate different features. In the left column the spectra are scaled to an arbitrary center-line height, while in the right column all spectra are scaled to reflect the same concentration of the spin label as in spectrum *a*. This procedure allows direct comparison of the spectral line shapes.

At the lowest lipid content (Fig. 2*a*), the ESR spectrum is characteristic of strong immobilization of the spin labels. As the lipid content of the membrane increases, a second, more mobile spectral component is easily distinguished (see *dashed arrow* in spectrum *b*). With further increase in lipid content, the relative intensity of the more mobile feature increases until the bound component is completely obscured (spectrum *d*). This is particularly evident when these spectra are scaled to the same concentration (Fig. 2, right). Because of the narrow line width, the line heights of the mobile component are large and are off scale in spectra *b*–*e*, Fig. 2, right.

The ESR data of Fig. 2 point out three interesting facts: (*i*) the presence of strongly immobilized spin label at low lipid levels, (*ii*) the appearance of a very much more mobile component in membranes with higher amounts of lipid, and (*iii*) the composite nature of the spectra associated with intermediate lipid content. We will now consider each of these facts separately.

In spectrum *a* of Fig. 2, the *arrows* indicate the position of the outer hyperfine extrema. In the absence of molecular motion (e.g., at low temperature) the distance between these two extrema is $2A_{zz}$, where A_{zz} is the principal value of the electron–nuclear hyperfine interaction measured along the nitroxide *z* axis. As molecular motion increases, these outermost lines move in, so that a measurable spectral feature, $2A_m$, serves as an indicator of relative molecular motion. The value of $2A_m$ for spectrum *a* (measured between the two *solid arrows*) is 64 G; the same sample at 77° K has $2A_m = 2A_{zz} = 69$ G. Since $2A_m$ is very nearly as large as $2A_{zz}$, it is apparent that *the spin label in cytochrome oxidase with low lipid content is almost completely immobilized.*

In contrast to the strong immobilization seen in spectrum *a* of Fig. 2, the spectrum of the spin label in lipids alone is characteristic of a fluid environment (spectrum *e*). In previous studies, similar line shapes were observed when the same spin label was present in liposomes of egg lecithin. Thus, the degree of fluidity of the lipid bilayers composed of cytochrome oxidase lipids is comparable to that of egg lecithin bilayers. This spectrum serves as a useful reference with which the composite spectra may be compared. The ^{14}N splitting constant measured from the mobile components of spectra *b*–*d* are 14.2 ± 0.2 G, a value typical of hydrocarbon environments. The same spin label in phosphate buffer has a substantially larger splitting constant (*i.e.*, 15.5 ± 0.2 G). This solvent effect and the line shape rule out the possibility that the mobile component arises from spin label in the aqueous phase. Clearly the mobile component present in spectra *b*–*d* is similar to spectrum *e*. Based on this evidence, we can conclude that *at higher lipid/protein ratios, cytochrome oxidase membranes contain fluid lipid bilayer regions.*

Furthermore, it is clear from Fig. 2 that there are two spectral components present, rather than a continuum. *Thus, two distinct lipid environments are present in cytochrome oxidase membranes.*

Quantitative spectral analysis

The spectra of Fig. 2 suggest that two distinct phospholipid environments are present in this model membrane system. Three interesting questions arise at this point. (*i*) Do all spectra of Fig. 2 represent combinations of the *same* two spectral components? (*ii*) If so, what are the ratios of the spin-label concentrations giving rise to the two components? (*iii*) Are the number of immobilized lipid sites constant over a wide phospholipid range? The questions can be approached by summation of various proportions of the putative components. For this purpose, the individual components were assumed to be the spectra from the cytochrome oxidase sample of lowest lipid content (0.10 mg of phospholipid per mg of protein) and from the sample of cytochrome oxidase lipids dispersed in buffer (Fig. 3, spectra *a* and *c*, respectively). These two spectra were digitalized, aligned horizontally with external *g*-value references, and then summed in different proportions to give the synthesized spectra *b*, *c*, and *d*, Fig. 3. These summed spectra are a good approximation of the corresponding experimental spectra (Fig. 2, *b*, *c*, and *d*). The composite spectra do indeed appear to be approximated by various combinations of the same two spectral components.

A digitalized spectrum can easily be integrated twice (17) to determine the relative concentration of spin label. In the summed spectra the relative concentrations were determined by integration of the individual components. By this procedure, i.e., summing to simulate experimental composite spectra and then integrating to determine relative contributions of each component, the proportions shown at the left of Fig. 3 were calculated. Thus, it is possible to estimate the relative spin-label concentrations contributing to the composite spectrum.

The third question concerns the amount of immobilized phospholipid relative to the amount of protein present. Assuming the distribution of the lipid spin label faithfully reflects the distribution of phospholipids in the two environments, the amount of bound lipid, C_b, in units of mg of phospholipid per mg of protein, is simply $C_b = C_t\chi$, where C_t

is the experimental value for total phospholipid of the membrane preparation in units of mg of phospholipid per mg of protein and χ is the fraction of the total absorption contributed by the bound component. From Fig. 3, the values of χ for the three membrane preparations (Fig. 3, *b*, *c*, and *d*) are 0.95, 0.69, and 0.34, respectively. Therefore, the corresponding calculated values of C_b are 0.23, 0.23, and 0.17 mg of phospholipid per mg of protein. These values of C_b are remarkably similar, considering the approximations involved, and we conclude that *the amount of phospholipid bound to the protein is independent of the extent of the fluid bilayer region.* Membranes formed of cytochrome oxidase thus appear to require about 0.2 mg of phospholipid for maximum occupancy of protein-bound sites.

We have also determined C_b by spectral subtraction of the bound component from each of the spectra, as well as by the summation technique, and also by solving simultaneous equations derived by subtraction of one composite spectrum from another. The range of values for C_b arrived at with these three techniques is 0.17–0.23 mg of phospholipid per mg of protein, confirming that $C_b \simeq 0.2$ mg of phospholipid per mg of protein. It is possible, of course, to have less than full occupancy of the bound sites. For example spectrum *a* of Figs. 2 and 3 contains 0.10 mg of phospholipid per mg of protein, and only 50% of the sites are occupied ($C_b = 0.1$). Preparations where $C_t \leq 0.19$ show only the bound component, whereas, when $C_t \geq 0.24$, the spectra also show a mobile component (Fig. 2). Evidently, as the lipid content increases, the bound sites are fully occupied, and phospholipid in excess of this amount forms fluid bilayers.

Estimation of boundary lipid

Given the observation that about 0.20 mg of phospholipid per mg of protein is immobile in the cytochrome oxidase membranes, the obvious question arises as to how this can be accounted for in molecular terms. The immobilization is evidently an effect of the protein on the lipid, since no counterpart is found in the spectrum of the lipids extracted from the membranes.

As observed by electron microscopy, the protein complexes have an irregular shape in the plane of the membrane (18). This shape can, however, be approximately represented by a rectangle of 52 × 60 Å, based on measurements made on the micrographs. A plausible hypothesis is that the first lipid layer around the protein complex corresponds to the immobilized component. We can calculate the number of lipid molecules that can be accommodated in a layer that is one aliphatic chain thick and then compare the value obtained with the measured amount found.

The diameter of an extended aliphatic chain is about 4.8 Å, as deduced from x-ray diffraction studies of hexagonal close-packed arrays of phospholipids (19). The perimeter of a rectangle 52 × 60 Å equals 224 Å. Division of the perimeter by 4.8 Å yields 47 aliphatic chains. This number must be divided by 2 to get the number of equivalent phospholipid molecules, but must also be multiplied by 2 since the bilayer arrangement is assumed, giving 47 first-layer phospholipids per protein complex. (An equivalent phospholipid molecule is defined to contain one phosphorous atom and 2 aliphatic chains. On this basis, one real molecule of cardiolipin corresponds to 2 equivalent phospholipid molecules.) From the molecular weights used for the protein complex (i.e., 210,000) and the phospholipid molecules (i.e., 775) (18), the result is obtained that 0.17 mg of phospholipid per mg of protein can be accommodated in the first layer around one protein complex. This is, of course, an approximation; the irregular real perimeter of the protein would tend to increase this value, while protein–protein contacts would tend to decrease it. Also, the first layer was defined to consist of one layer of aliphatic chains; the immobilization effect might extend somewhat beyond that. With these reservations in mind, we note that *the amount of lipid that can be accommodated in the first (boundary) layer as calculated is very close to the observed amount immobilized.*

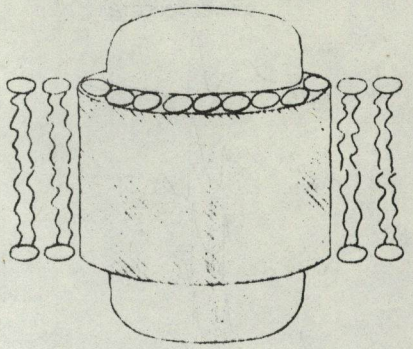


FIG. 4. Diagrammatic representation of a single protein complex and associated phospholipid in membranous cytochrome oxidase.

This hypothesis is summarized diagrammatically in Fig. 4, where the protein complex of membranous cytochrome oxidase is shown extending through the phospholipid region. The first layer of phospholipid is indicated as *boundary lipid* surrounding the hydrophobic regions of the protein complex. Beyond the boundary lipid is the more-fluid phospholipid bilayer. This model emphasizes the two lipid regions, boundary lipid and fluid bilayer, but oversimplifies the structure in that any protein–protein contacts are not shown and the demarcation between boundary lipid and fluid bilayer regions is exaggerated. The details of the lipid–protein interaction are unknown. However, the binding surfaces of the protein complex must be irregular, since the hydrophobic side groups of the polypeptide chains form the potential wells responsible for immobilization of the lipid spin labels.

The hypothesis of boundary lipid is adequate to explain the immobilization data. Another conceivable explanation is that the bound lipids are distributed more or less uniformly throughout the protein complex. Although there may be a few phospholipid molecules surrounded by protein, two observations argue against significant amounts of bound phospholipid *within* the protein complex. First, Vanderkooi *et al.* (18) have shown that measurements from electron micrographs (of crystalline regions that occur in some preparations of membranous cytochrome oxidase) are consistent with the interpretation that all phospholipid is distributed between the protein complexes. Second, the spin labels are observed to exchange between the boundary layer and fluid bilayer regions. This is most evident in the second column in Fig. 2. In this figure, the ratio of spin label to protein is held constant, while the total phospholipid increases from 0.10 to 0.49 mg of phospholipid per mg of protein. The height of the immobilized spectral component decreases monotonically as a func-

tion of mercuric phospholipid, indicating the ability to exchange between regions where the labels are immobilized and regions of fluid bilayer. Therefore, although we cannot completely rule out the possibility of phospholipid buried in the protein, the ESR data are most easily accounted for in terms of the boundary lipid model (Fig. 4).

In summary, we have shown that the model membrane formed by cytochrome oxidase and phospholipid contains two distinct phospholipid regions, differing markedly in fluidity. We interpret our data to support the concept that protein complexes extend into or through the bilayer region and that the hydrophobic protein surface tightly binds a layer of lipid, effectively reducing the phospholipid participating in fluid bilayer formation. Given the amphipathic properties of many membrane proteins, the existence of boundary lipid may be a general phenomenon and would tend to reduce the amount of phospholipid available to form fluid bilayer regions in biological membranes.

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