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Estimation of Lipid Regions in a Cytochrome Oxidase–Lipid Complex Using Spin Labeling Electron Spin Resonance: Distribution Effects on the Spin Label

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Abstract

The distribution of lipid in the cytochrome oxidase–lipid complex from beef heart mitochondria has been studied by the spin labeling electron spin resonance technique. The spectra of a phospholipid spin label incorporated in the complex reveals an immobilized (on the ESR time scale) component in addition to the fluid component which is found in aqueous dispersions of the extracted lipids. The first component corresponds to the domain of lipid influenced by the protein, and the second component to the remaining lipid. A theory taking into account not only the sizes of the lipid regions in which the spin label molecule distributes itself, but also the different affinities of the label for the two domains, has been developed. Taking advantage of the variation in spectra obtained with increasing amounts of spin label, computer calculations have been performed to estimate the distribution of lipid in the different regions of the cytochrome oxidase–lipid complex. An extrapolation of the amount of immobilized spin-labeled phospholipid to zero concentration of label allows a calculation of the number of fatty acid residues interacting with the protein to be made. It has been found that the number of aliphatic chains influenced by the protein is higher than that calculated for a single boundary layer around the protein. The approach used in this paper can be useful for studies of protein–lipid interactions in other systems.

Key Words: Protein–lipid interactions; cytochrome oxidase; spin label.

Introduction

Studies of protein–lipid interactions in biological membranes are often difficult to interpret partly due to the complexity of the many interactions

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taking place. One possible simplification is to isolate and study a complex (for example, a protein with its accompanying phospholipids) from a membrane.

Cytochrome oxidase isolated from mitochondrial membranes provides an interesting model for studies of the association of lipid with protein in a functional complex. The usefulness of this model has already been shown by Griffith *et al.* (1973) and Jost *et al.* (1973a, b, c; 1977), who have used spin-labeled fatty acids and phospholipids to study protein–lipid interactions in the purified cytochrome oxidase–lipid complex. The ESR spectra showed two components which have been interpreted as arising from two lipid environments: (1) a layer of phospholipids coating the hydrophobic protein surface (the so-called boundary layer) and (2) the remaining fluid phospholipid bilayer. The arrangement has been suggested to be a general feature of all biological membranes. Marsh *et al.* (1978), studying the protein-immobilized lipid in dimyristoylphosphatidylcholine-substituted cytochrome oxidase, demonstrated a strong perturbation of the lipid-bilayer fluidity which was quite distinct from the immobilized boundary layer. On the other hand, Seelig and Seelig (1978) and Rice *et al.* (1979) did not obtain any evidence for a long-lived boundary layer of immobilized lipids in reconstituted cytochrome oxidase phospholipid membranes studed by deuterium and ^{31}P nuclear magnetic resonance. These authors pointed out the limitations of conventional spin label ESR resulting from the fairly narrow frequency range, giving a tendency for conventional ESR to focus on the short-lived transition state of the lipid–protein interactions. The following abbreviations are used: ESR, electron spin resonance; NMR, nuclear magnetic resonance; PSL, phospholipid spin label.

Motions with frequencies slower than 5×10^6 Hz will give rise to an immobilized spectra even though there can still be an exchange taking place of spin-labeled phospholipid molecules between the surface of the protein and the bulk lipid.

Despite the limitations of the spin label method, ESR spectra of spin-labeled phospholipids in cytochrome oxidase give some information about the lipid surrounding the protein molecule and the bulk lipid. Jost *et al.* (1973a, b, c) have assumed that the partition of the spin label faithfully reflects the distribution of lipid between the two domains (the boundary layer and the fluid bilayer). The amount of spin label giving rise to the two components of the ESR spectra has been considered to reflect the size of the two domains of lipid, but no theoretical basis has been presented for this approach.

In this paper we have studied purified cytochrome oxidase from beef heart mitochondria, without lipid extraction (i.e., a soluble functionally active complex), using a phospholipid spin label incorporated into the complex. A

theory which allows an estimate to be made of the amount of lipid associated with the complex is presented. One region is the domain of lipid (immobilized *on the ESR time scale*) surrounding the protein and influenced by the protein. The second is the remaining lipid which on the same time scale does not exhibit a restricted molecular motion. The theory developed in this paper takes into account not only the size of the domains in which the spin-label molecule distributes itself, but also the different affinities of the label for the domains. Taking advantage of the variation in spectra obtained with increasing amounts of spin label, we present computer calculations made to estimate the extent of the lipid regions or domains in the cytochrome oxidase–lipid complex. It has been found that the number of aliphatic chains influenced by the protein is higher than can coat the hydrophobic surfaces of the protein in a layer that is one aliphatic chain thick.

Materials and Methods

Chemicals

Spin-labeled phospholipid (PSL) was synthesized by Dr. A. Elias (Department of Chemistry, Chelsea College, University of London) from egg yolk lysolecithin and palmitic acid, spin-labeled in position 10, according to the method of Boss *et al.* (1975). Analytical grade reagents were used.

Preparation of Cytochrome Oxidase and Lipid Extraction

Cytochrome *c* oxidase was prepared from beef heart mitochondria essentially as described by Kuboyama *et al.* (1972) with Tween 80 substituting for Emasol. The final preparation was dialyzed against 30 mM potassium phosphate buffer pH 7.0 and contained 20 mg protein/ml.

Cytochrome oxidase lipids consisted of the pooled supernatants from successive 30-min extractions with cold 90% aqueous acetone (Fleischer and Fleischer, 1967), followed by centrifugation, evaporation of the solvent, and suspension in distilled water. Prior to each extraction the suspensions were sonicated for 2 min (low-power sonication, on ice, in periods of 15 s at 30-s intervals).

Fatty Acid Analysis

The fatty acid composition of purified cytochrome oxidase and of lipids extracted from cytochrome were analyzed as follows. Fatty acids were methylated in a solution of 14% BF_3 in methanol by heating at 70°C for 2

min. The methyl esters were extracted into light petroleum ether, dried over anhydrous Na_2SO_4, and separated by gas chromatography in a Pye series 104 gas chromatograph using a stationary phase of 10% polyethylene glycol adipate on a support of Chromosorb Q (100–120 mesh) with a carrier gas of nitrogen and a temperature of 197°C. Retention times of authentic fatty acid methyl esters were used to identify fatty acids. Heptadecanoic acid was added in the initial sample (before methylation) and run as an internal standard. The concentration of the fatty acids was calculated using the peak area of the internal standard.

Spin Labeling and ESR Measurements

The phospholipid spin label (PSL) was dissolved in chloroform and various aliquots were dried with a stream of nitrogen. Cytochrome oxidase suspension (50 μl) was added to each sample and the mixture was dispersed using a bench vibrator for several minutes. ESR spectra were obtained with a Varian E4 spectrometer (9.5 GHz, 100-kHz field modulation). Samples were sealed in melting point tubing and supported vertically in a narrow quartz tube.

Computer Spectral Analysis

The experimental first-derivative ESR spectra were digitalized and aligned horizontally with reference to g-value markers. The baselines of the spectra were adjusted to pass through the weighted average of the points, and the spectra were normalized to unit doubly integrated area and stored for future replotting, lineshape subtraction, or addition by computer. The computer program was an extension of the package of algorithms for ESR lineshape synthesis already described (Porumb and Slade, 1976a, b). The quantitative analysis of the experimental ESR spectra followed the conventional methods of titration by subtraction and summation of the spectral components. The proportion of mobile and immobile components in a composite sample was given by the proportion of the spectra of the two components, normalized to unit doubly integrated area, which made up the composite lineshape.

Theoretical Model

The classical partition coefficient k, which measures the relative solubility of a substance in the two phases of a binary system, is defined mathematically by Eq. (1). The equation is written for the particular case where the two phases are the mobile and "immobilized" lipid domains of a lipid–protein

complex:

$$k = \frac{\text{molecules partitioned to the "immobilized" domain} \,/\, \text{magnitude of the "immobilized" domain}}{\text{molecules partitioned to the mobile domain} \,/\, \text{magnitude of the mobile domain}} \tag{1}$$

In the classical concept k is a constant, independent of the amount of substance used.

The following two assumptions are made:

(i) The number of lipid molecules per protein molecule showing hindered hydrocarbon chain motions as a result of interactions with the protein, denoted by b, is a constant, irrespective of the type of lipids which constitutes this domain, i.e., b includes both labeled and unlabeled molecules. The magnitude of the "immobilized" domain, b, can be constant if there is an exchange competition between labeled and unlabeled lipid molecules, i.e., a labeled lipid molecule becomes "immobilized" by displacing an "immobilized" native lipid molecule from the neighborhood of the protein.

(ii) The partition coefficient (k) for the labeled lipid molecules in the "immobilized" lipid/mobile lipid binary system is constant, independent of the amount of labeled lipid used. This may mean that the difference in the affinities (treated here as solubilities) of the labeled lipid molecules for the two domains arises from the difference in the mobility of the respective domains, in all other respects the labeled and unlabeled molecules being equivalent.

One may thus write

$$k = \frac{n_i^*/b}{n_m^*/(n + n^* - b)} = \text{const} \tag{2}$$

where n_i^* and n_m^* are the number of labeled lipid molecules per protein molecule partitioned to the "immobilized" and the mobile domain, respectively, $n^* = n_i^* + n_m^*$ is the total number of labeled lipid molecules per protein molecule, and n is the number of unlabeled, native lipid molecules per protein molecule in the sample.

By cross multiplying the members of Eq. (2) and applying elementary properties of the proportions, one obtains the equivalent form [Eq. (3)], which is the functional relationship sought:

$$\frac{n^*}{n_i^*} = \frac{n^*}{kb} + \frac{n - (1 - k)b}{kb} \tag{3}$$

According to Eq. (3), the reciprocal of the fraction of "immobilized"

labeled lipid molecules, n^*/n_i^*, which is the information obtained from the ESR spectral analysis, is expected to vary linearly with n^*, the labeled lipid to protein ratio, i.e., the graph of n^*/n_i^* vs. n^* is expected to be a straight line. To verify the validity of this relationship one thus needs the results of the ESR spectral analysis from a set of samples with variable amounts of labeled lipid.

Finally, the two unknown quantities, k and b, can be calculated from the experimentally determined slope S and intercept I of the straight-line graph as

$$k = 1/(nS - I + 1) \tag{4}$$

$$b = 1/(kS) \tag{5}$$

The native lipid to protein ratio n can be obtained by chemical analysis.

The linear extrapolation to zero label according to Eq. (3) should provide an image of the lipid–protein system as seen by the spin label under conditions of minimum interference from the label itself. The expression for the intercept of the graph is formally obtained from Eq. (3) by letting the total number of labeled lipid molecules n^* tend to zero. One obtains

$$I = \frac{n - (1 - k)b}{kb} \tag{6}$$

If the partition of the spin label reflected faithfully the proportion of the magnitudes of the two domains, one would expect the right-hand side of Eq. (6) to be equal to the reciprocal of "immobilized" native lipid in the original sample, n/b. However, the magnitude of the "immobilized" domain, b, appears in the denominator of Eq. (6) multiplied by the fraction k, while the total number of native lipid molecules, n, which appears in the numerator, is decreased by the complementary quantity $(1 - k)b$. Thus the system behaves as if the label, while reporting all the lipids of the mobile domain, can only "see" a well-defined fraction of the lipids of the "immobilized" domain (kb), the remaining $(1 - k)b$ being ignored. Therefore a consequence of the labeled lipid having different affinities for the two domains is that the label does not report correctly the magnitude of the "immobilized" domain.

Results and Discussion

Characterization of the Purified Cytochrome Oxidase

The enzyme had a heme aa_3:protein ratio between 4 and 5 μmol g^{-1} and maximal turnovers (electrons per aa_3) of 100–200 s^{-1} in the presence of 0.04% asolectin, 67 mM potassium phosphate, pH 7.4, with ascorbate and horse cytochrome c. The purified cytochrome oxidase was 270 ± 50 μg

lipid/mg protein according to the estimation based on gas chromatographic fatty acid analysis (which gave 209 μg fatty acid/mg protein). The results of the fatty acid analysis of the purified cytochrome oxidase and of the lipids extracted with acetone are shown in Table I. The major part of the lipid consisted of the detergent Tween 80 (an oleate ester) as shown by the high content of oleic acid in the lipid extract as well as in the cytochrome oxidase. It can be seen that fatty acids occur in a similar proportion in the acetone extract as in the purified oxidase. No preferential extraction of any of the fatty acids could be noticed, all being extracted by approximately 50–60%.

Taking into account the molecular weights of phospholipid and of the detergent Tween 80 the data in Table I can be converted to moles of phospholipid and of detergent per mole of cytochrome oxidase. If the assumptions are made of a molecular weight of 210,000 for the oxidase (Griffith *et al.*, 1973) and of molecular weights of 775 and 340 for the phospholipids and Tween 80 respectively, then the purified cytochrome oxidase contains approximately 16 moles of phospholipid and 132 moles of detergent per mole of protein. From these about 10 moles of phospholipid and 83 moles of detergent per mole of protein are acetone extractable.

Spin-Labeled Cytochrome Oxidase

Representative spectra of PSL dispersed in the purified cytochrome oxidase (spectra A–H) and in liposomes prepared from cytochrome oxidase

Table I. Fatty Acid Composition of Cytochrome Oxidase and of Lipid Extracted from Cytochrome Oxidase

	Cytochrome oxidase		Acetone-extractable lipid	
Fatty acid	μg/mg protein	% of total	μg/mg protein	% of total
16:0	10.5	5.22	6.2	4.77
16:1	13.8	6.60	8.2	6.31
18:0	1.4	0.66	1.0	1.14
18:1	168.7	80.7	105.9	81.46
18:2	3.4	1.62	2.5	1.92
20:0	5.3	2.53	2.6	2.00
20:1	5.9	2.82	3.6	2.77
Total fatty acid (lipid plus detergent)	209		130	
Total fatty acid (phospholipid)	40.3		24.1	

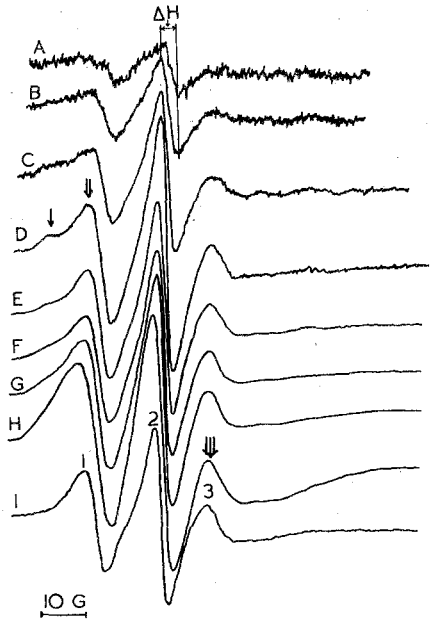

Fig. 1. ESR spectra obtained with increasing amounts of phospholipid spin label in the cytochrome oxidase dispersion. Spectra were recorded with modulation amplitude 1.0 G and microwave power 0.48 mW. (A) 3 nmol PSL/mg protein, gain 10.0×10^3; (B) 15 nmol PSL/mg protein, gain 8.0×10^3; (C) 30 nmol PSL/mg protein, gain 6.3×10^3; (D) 60 nmol PSL/mg protein, gain 1.6×10^3; (E) 150 nmol PSL/mg protein, gain 1.6×10^3; (F) 300 nmol PSL/mg protein, gain 10.0×10^2; (G) 600 nmol PSL/mg protein, gain 8.0×10^2; (H) 1500 nmol PSL/mg protein, gain 8.0×10^2; (I) spectrum of PSL in liposomes from the lipids extracted from cytochrome oxidase.

lipids (spectrum 1) are shown in Fig. 1. The spectra are arranged in order of increasing PSL content of the samples. Several interesting qualitative observations can readily be made from these data. First, a composite spectrum having two components (a mobile and an immobile one) occurs for the PSL dispersed in the purified cytochrome oxidase lipids. This is readily seen in spectrum D where the double arrow indicates the appearance of the mobile component superimposed on the immobilized spectrum (indicated by the single arrow). Second, when different ratios of spin-labeled molecules to protein are used, the proportions of the mobile and immobile components of the spectra vary considerably. At low PSL content of the sample the spectra

indicate a relatively high proportion of immobilized spin label (spectra A–C). At higher PSL contents the mobile component of the spectrum increases (spectra D–F). This mobile component bears a close resemblance to the spectrum obtained from the isolated lipids (spectrum 1). At very high PSL content (spectra G and H) strong interactions (dipole–dipole and spin exchange) between the probe molecules occur. This is shown by a characteristic increase in AH (the peak-to-peak distance of the central band indicated in Fig. 1), a decrease in the high-field peak height of the inner hyperfine doublet (triple arrow in Fig. 1, spectrum H), and the downward displacement of the high-field baseline. Spectra G and H have not therefore been subjected to further analysis by computer. Spectrum A, where the signal-to-noise ratio is very low, has also not been used for quantitative analysis.

Quantitative Estimation of the Spectral Components by Computer Analysis: Lipid Regions in Cytochrome Oxidase

The spectrum of the mobile component was provided by the sample of PSL diffused into liposomes prepared from the lipids extracted from the purified cytochrome oxidase (spectrum I, Fig. 1). The spectrum of the immobile component was provided by a sample of a phospholipid spin label bound to albumin in solution (see Fig. 2a in Benga and Chapman, 1976).

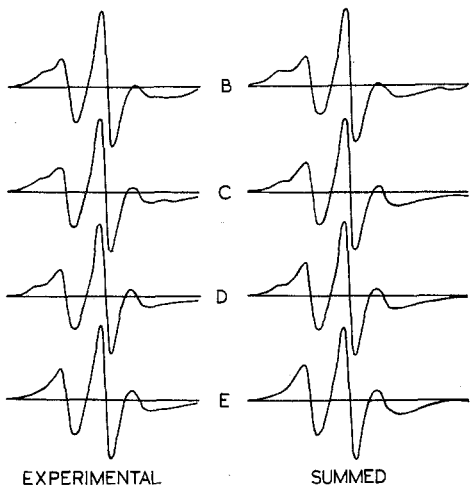

Fig. 2. The experimental spectra of PSL in cytochrome oxidase dispersion normalized to center peak height and the corresponding synthesized spectra obtained by summation of the mobile and immobile component spectra in different proportions as described in the text. The notation of spectra is that of Fig. 1.

Two methods of spectral analysis have been used. One is the spectral titration by subtraction, in which difference spectra are obtained by subtraction of one component from the composite experimental spectrum. The endpoint has been judged by the disappearance of that component, by the overall lineshape, and by the appearance of phase reversal of the component. The relative proportion of the mobile and immobile PSL components in the sample B–D (Fig. 1) was estimated by carrying out spectral titration against either of the component spectra.

The results were checked using a second method, based on the summation of the mobile and immobile content spectra in different proportions to match with the experimental composite spectra. The sums obtained using different ratios of the components gave synthesized spectra that were good approximations to the experimental spectra (Fig. 2). This analysis gives confidence to the interpretation that the spectra of Fig. 1 do represent summations of the same two spectral components.

The results of both analytical approaches, subtraction and summation, on the spectra of samples with increasing PSL content are given in Table II. The agreement in the amount of the immobile component obtained by the two methods of spectral analysis is good, taking into account the difficulties encountered in judging exact endpoints. Although both methods rely on the visual examination of lineshapes, the summation procedure enables more accurate decisions to be made, as the spectra obtained by this method were free of the "noise" sometimes generated by the subtraction procedure. The consistency of the results was also checked by subtractions of the experimental spectra in pairwise fashion.

The results of spectral analysis have shown that, using increasing amounts of spin-labeled phospholipid added to a certain amount of cytochrome oxidase, the proportion of the immobile component of the spectra decreases considerably (from 50% in spectrum B to 13% in spectrum D in Fig. 1). If the data are plotted as the reciprocal of the percentage of immobilized PSL versus the amount of spin label in the sample, the points approximately

Table II. Proportion of the Immobile Component in the Spectra from Fig. 1

	% Immobile component[a]		
Spectrum	Titration against mobile spectrum	Titration against immobile spectrum	Summation of spectral components
B	45 ± 5	60 ± 10	50 ± 5
C	33 ± 8	45 ± 10	36 ± 6
D	29 ± 5	29 ± 5	29 ± 5
E	13 ± 5	16 ± 8	13 ± 1.8

[a]The figure indicated for each procedure corresponds to the best approximation to the experimental spectra.

lie on a straight line (Fig. 3). An extrapolation to zero concentration of the label gives a proportion of 75 immobilized labeled lipids in the cytochrome oxidase–lipid complex, the remainder being in a mobile phase. The immobile and mobile components of the spectrum of spin-labeled cytochrome oxidase–lipid complex are usually considered (Griffin *et al.*, 1973; Jost *et al.*, 1973a, b, c) to correspond to two classes of lipids: (a) lipid tightly bound to the protein and (b) the lipids in a fluid bilayer. In previous studies (Jost *et al.*, 1973a, b, c) an equal partition of the spin-labeled fatty acid between the fluid and rigid regions has been assumed. However, no direct evidence for such an assumption has thus far been provided. On the contrary, spin-labeled lipids have been found in several model lipid systems to partition preferentially into the more fluid phase (Butler *et al.*, 1974; Kuboyama *et al.*, 1972). We have shown (Benga and Chapman, 1976) that there can be some uncertainty regarding the distribution of spin-labeled fatty acid in a protein–lipid system, so that it is necessary to be cautious about quantitative estimations of the state of lipids using only spin-labeled fatty acids. On the contrary, more reliable data are obtained using spin-labeled phospholipids. For this reason we have chosen a phospholipid spin label to study the lipid regions of the cytochrome oxidase–lipid complex. As previously mentioned in this paper the immobile component of the spectrum should not be considered to correspond to the lipid tightly bound to protein, but to the lipid that *on the ESR time scale* is influenced by the protein.

Another criticism of the spin-label technique is the uncertainty as to whether the probe detects average or local membrane properties. Only a spin probe uniformly distributed throughout the membrane would represent the fluid and immobilized lipid of mixed systems in proportion to their relative

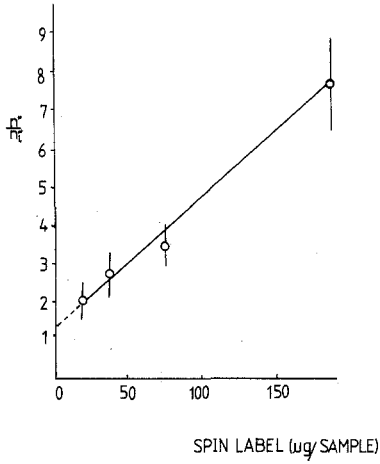

Fig. 3. Plot of the reciprocal of the fraction of immobilized spin-labeled lipid (n/n_i) against the amount of spin label added to the sample. The bars correspond to the estimated error from Table II.

concentrations. Bieri *et al.* (1974) interpret the spin-labeled fatty acid-induced perturbation of the erythrocyte membrane in terms of segregation of the probe in the membrane; the presence of high local concentrations of probe is indicated by the noticeably exchange-broadened spectra. It has been considered that probe–probe interactions such as these may obscure the nature of "intrinsic" membrane properties. The PSL concentration effect on the ESR spectra of the cytochrome oxidase–lipid complex described in this paper may be interpreted on the basis of the above discussed findings. Adding more spin-labeled molecules causes the spectrum to appear more mobile (Fig. 1). The spectra A–D in Fig. 1 indicate the progressive partition of the label into the fluid phase, presumably situated farther away from the protein and consequently less immobilized on the ESR time scale. This interpretation is in agreement with the preference of spin labels for the most fluid areas in a heterogeneous environment. In addition, the exogenous lipid probe may itself influence the properties of the lipid environment in which it resides. Because of this, high PSL/lipid ratios result in exchange-broadened spectra (spectra G and H in Fig. 1). These are due to strong probe–probe interactions. It is clear that the preferential partition of label molecules into the fluid lipid and the strong interactions occurring at high concentrations of probe may obscure any "intrinsic" membrane properties (Sauerheber *et al.*, 1977), rendering the quantitative estimation of the proportions of lipid regions in cytochrome oxidase difficult. Sauerheber *et al.* (1977) have shown that the "intrinsic" membrane properties may be evaluated only if probe concentrations in the "low" range are used: these authors criticize a number of ESR studies of membrane systems, including the work of Jost *et al.* (1973a, b, c), because probe concentrations in the "high" range were used. "Intrinsic" properties are defined by Sauerheber *et al.* (1977) as those which are measured when probe–probe interactions are negligible, but not, however, in the total absence of a perturbing spin label. We think that one should try to find the "intrinsic" properties of a membrane system at zero concentration of label. The large variations of the proportions of the immobile and fluid components of the spectra of PSL diffused in the purified cytochrome oxidase complex make the extrapolation to zero concentration of label possible. The theory developed in this paper justifies this extrapolation.

In applying the quantitative theory, account was taken of the fact that the Tween 80 detergent consisted of one aliphatic chain, while both the native and labeled phospholipids had two aliphatic chains. In the calculations a phospholipid molecule was thus considered to be equivalent to two detergent molecules, and it was assumed that both chains of the phospholipid molecule took part in the interaction with the protein, i.e., a labeled phospholipid molecule, in order to become "immobilized," displaced either one native

phospholipid molecule or two detergent molecules from the neighborhood of the protein.

A least-squares treatment of the data obtained from the quantitative spectral analysis (Table II) yielded the following values for the slope and the intercept of the linear graph (Fig. 3):

$$S = 4.4 \times 10^{-2} \pm 0.3 \times 10^{-2} \text{ moles protein/mole phospholipid}$$

$$I = 1.3 \pm 0.2$$

The abscissas of the points were previously converted to moles labeled phospholipid/mole protein, using the information given in the Materials and Methods section.

Application of formula (4) led to a value of the partition coefficient for the labeled phospholipid in the "immobilized":mobile binary system equal to $k = 0.3 \pm 0.04$, i.e., the affinity of the labeled lipid for the mobile domain was about three times higher than that for the "immobilized" one. This also means, according to the considerations made in the theoretical model, that the labeled phospholipid only reported about 30% of the number of lipids of the "immobilized" domain, the remaining ones being, in the absence of adequate calculations, ignored.

For the magnitude of the "immobilized" domain, application of formula (5) yielded the value $b = 75 \pm 6$ moles phospholipid/mole protein or 150 ± 12 "immobilized" aliphatic chains per protein molecule. This corresponds to about 14.5 phospholipid molecules (29 chains) and 121 detergent molecules per molecule of cytochrome oxidase, representing about 92% of the total number of aliphatic chains in the original sample.

Jost *et al.* (1973a, b, c) present estimates of how the lipid immobilized by cytochrome oxidase can be accounted for in molecular terms. They assume approximate dimensions of the protein complex (in the membrane plane) of 52×60 Å and the diameter of one aliphatic chain of 4.8 Å and calculate that about 47 molecules of phospholipid would be necessary to coat the protein by a bound lipid layer that is one aliphatic chain thick. The 47 molecules of phospholipid would give about 0.2 mg phospholipid/mg protein, and this is the amount of lipid estimated by them to be immobilized in the purified cytochrome oxidase.

The approach by Jost *et al.* (1973a, b, c) was to gradually reduce the phospholipid content of the purified cytochrome oxidase complex by successive aqueous acetone extractions. It can be argued that by extraction with acetone the organization of lipids in the complex is changed and the structure of protein is irreversibly modified. Jost *et al.* (1977) have also used alternative methods to study purified cytochrome oxidase preparations of various lipid content using spin-labeled phospholipids. They report the same amount of

phospholipid to be immobilized by the protein as in their previous work, corresponding to one layer of phospholipid coating the hydrophobic surface of protein. However, no mention is made in any of this work of the amount of detergent present in their preparations which were isolated in Triton X-100. The cytochrome oxidase complex studied in the present paper has been characterized in some detail with regard to the composition of purified cytochrome oxidase lipid. Moreover, the gas chromatographic analysis of fatty acids allows us to estimate the amount of the detergent Tween 80 in the cytochrome oxidase complex. Tween 80 lacks any other quantitative assay. The possible molecular arrangement of both phospholipid and detergent molecules is still not clear. Assuming the same dimensions of protein complex (52 × 60 Å) and of diameter of one aliphatic chain (4.8 Å) as Jost *et al.* (1973a, b, c), the present results indicate that a greater number of aliphatic chains seem to be influenced by the cytochrome oxidase than can be accommodated in a simple boundary layer. It should be emphasized that almost all the lipid in the system appear to be influenced by the protein.

Seelig and Seelig (1978), using deuterium and ^{31}P nuclear magnetic resonance, have found that the incorporation of cytochrome oxidase into phosphatidylcholine bilayers leads to a more disordered conformational state of the lipids. They also conclude that the lipid–protein interactions are not limited to a monomolecular annulus; on the contrary, the protein is capable of influencing larger domains of the surrounding lipid.

The present results, obtained by a different approach, are in agreement with these conclusions. Moreover, it is clear that the theoretically based two-component analysis of the ESR spectra obtained from spin-labeled lipid in the cytochrome oxidase system yields a conclusion which opposes the concept of an immobilized boundary layer. The different affinities of the spin-label molecule toward the different lipid regions have to be considered in any analysis. It is interesting to note that a similar conclusion has been reached for another protein–lipid system by Marsh and Barrantes (1978). They report that in acetylcholine-receptor-rich membranes from *Torpedo marmorata* the proportion of lipid in the immobilized component is greater than that calculated for a single boundary layer around the protein.

Despite some inherent uncertainties of the spin-label technique, spin probes have proven to be valuable investigative tools for studies of membrane structure and function. However, the interpretation of ESR spectra of spin-labeled membranes in terms of the molecular organization of membrane components is not necessarily straightforward. The new approach to the study of protein–lipid interactions described in this paper using an extrapolation of immobilized spin-labeled phospholipid to the zero concentration of the label can be useful in studies of other systems.

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