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IDENTIFICATION AND EXTENT OF FLUID BILAYER REGIONS IN MEMBRANOUS CYTOCHROME OXIDASE

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SUMMARY

Membranous cytochrome oxidase isolated from mitochondria provides a useful model membrane for studies of hydrophobic lipid association with a fairly well characterized functional protein complex. In order to examine the lipid environments, isotropic and oriented samples of cytochrome oxidase of varying phospholipid content were spin labeled with 5-doxylstearic acid (the 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of 5-ketostearic acid), and examined by ESR spectroscopy. The isotropic samples exhibit two spectral components. Based on the relative intensity of the two components, the mobility, and the response to hydration, the two components are interpreted as arising from two lipid environments: (1) a layer of lipid bound to the hydrophobic protein surface (boundary lipid) and (2) fluid phospholipid bilayer regions. The extent of the bilayer regions is estimated from integration of the two components of the ESR spectra for samples with different phospholipid/protein ratios. The anisotropy of macroscopically oriented membranous cytochrome oxidase samples containing 0.33 and 0.49 mg phospholipid/mg protein confirms the presence of phospholipid bilayer regions. A spectral analysis reveals a remarkable similarity between the phospholipid bilayer regions in the cytochrome oxidase model membranes and bilayers formed by the isolated lipids, whereas the boundary lipid component has no counterpart in lipid bilayers.

INTRODUCTION

When enzymatically active cytochrome oxidase is isolated from the inner mitochondrial membrane it contains associated phospholipids and spontaneously forms closed membranous vesicles[1]. The interactions between the cytochrome oxidase–protein complex and the phospholipids are undoubtedly meaningful in relation to similar associations in the intact inner mitochondrial membrane. Thus, cytochrome oxidase membranes form a model membrane in which the hydrophobic association of a functional membrane protein with membrane phospholipids can be studied. The usefulness of this model system is enhanced by the fact that the phospholipid content can be varied[1] (see Fig. 1).

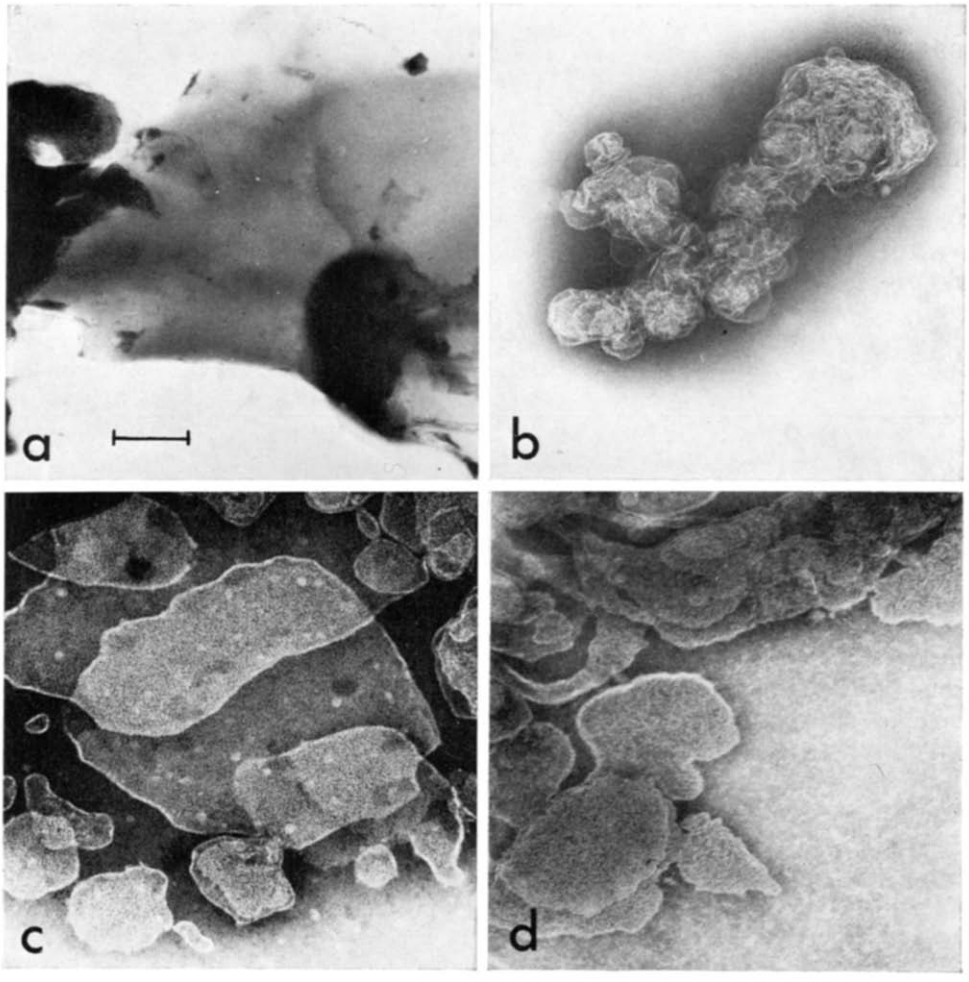
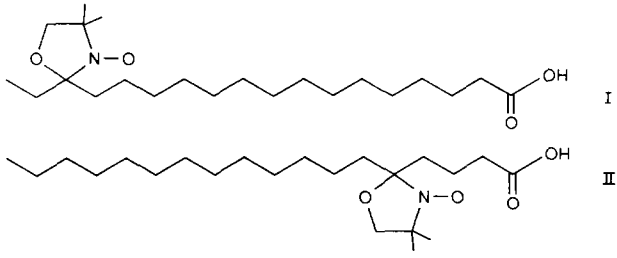


Fig. 1. Electron micrographs of negatively stained cytochrome oxidase samples of varying phospholipid content. The phospholipid content in mg phospholipid/mg protein is (a) 0.19, (b) 0.33, (c) 0.49 and (d) 0.73. Note the vesicular structure in b–d. At phospholipid/protein ratios of 0.24 or lower, cytochrome oxidase appears amorphous or sheet-like unless dispersed with detergent; at phospholipid/protein ratios of 0.33 or higher vesicular structures were seen. This closely resembles the preparations described by Chuang *et al.*[1] (magnification, a–d, bar equals 0.2 μm). Micrographs by Mr William Colquhoun.

In a previous spin-labeling study we have shown that a fraction of the phospholipid is bound to the hydrophobic surface of the cytochrome oxidase–protein complex[2]. The experimentally determined amount of phospholipid corresponding to maximum occupancy of protein-bound sites is about 0.2 mg phospholipid/mg protein. This bound lipid could be accounted for by a single layer of phospholipid (boundary lipid) surrounding the protein complex. At higher phospholipid/protein ratios a more mobile fraction was observed, which was presumed to be fluid bilayers. This study employed spin labeling and utilized 16-doxylstearic acid (I) (the trivial name for the 4′,4′-dimethyloxazolidine-*N*-oxyl derivative of the 16-ketostearic acid). The present study

is directed toward characterization of the presumptive bilayer regions and utilizes the 5-doxylstearic acid (II) spin label, which is better suited for the study of orientation and the effects of hydration.



Lipid spin labels of this type were introduced sometime ago for the study of membranes[3,4] and several reviews of spin labeling have been published[5–10].

MATERIALS AND METHODS

Cytochrome oxidase preparations

Cytochrome oxidase, with concentrations of 7.8–8.5 nmoles heme *a*/mg protein, was prepared from beef heart mitochondria by the general methods of Sun *et al.*[11] with minor modifications as described elsewhere[2]. The resulting membranous cytochrome oxidase preparations had a phospholipid content of 0.33–0.49 mg phospholipid/mg protein, assuming an average phospholipid mol. wt of 775. Additional phospholipid was incorporated by the method of Fleischer and Fleischer[12]. In order to obtain samples of reduced phospholipid content, successive cold 95% aqueous acetone extractions[12] were performed. The resulting cytochrome oxidase samples had phospholipid/protein ratios of 0.10, 0.15, 0.19, 0.24, 0.33, 0.49 and 0.73 mg phospholipid/mg protein, and all were active as estimated by the method of Smith[13]. Either the pooled lipids from the acetone extractions, or lipids extracted with chloroform–methanol (2:1, v/v) and subsequently extracted with chloroform, were used as representative of cytochrome oxidase lipids. Both lipid samples were found to contain no more than 7–10% protein. Lipids prepared by these two methods gave, after spin labeling, essentially identical ESR spectra. Phosphorous, protein, heme *a* determinations, assay of cytochrome oxidase activity and gel electrophoresis were performed as described elsewhere[2]. Preparations were examined by electron microscopy, using 1% phosphotungstic acid (pH 5) as the negative stain.

Preparation of spin-labeled samples

The spin label, 5-doxylstearic acid (Syva Associates), was evaporated from chloroform solution to form a thin film in a small glass vial. Buffered cytochrome oxidase (10 mM phosphate, pH 7.0) was added to the vial and stirred for 2 min by low power bath sonication (Heat Systems-Ultrasonics, Inc.), then allowed to stand on ice for 30 min before using. The labeling was kept constant at $2.5 \cdot 10^{-8}$ mole spin label/mg protein. Several drops of the labeled samples were then placed on slides made from microscope coverslips. After the cytochrome oxidase settled onto the glass slide most of the excess aqueous phase was removed by a fine capillary and then further reduced

by evaporation under nitrogen until only a thin film of moisture remained. Thin walled chambers, with a reservoir for water or aqueous salt solutions, were pre-equilibrated at the desired relative humidity and the spin-labeled cytochrome oxidase sample on the coverslip was inserted into the humidity chamber. Distilled water or aqueous salt solutions were used in the reservoir to attain the desired relative humidity as described earlier[14]. The closed chamber allows equilibration with constant relative humidity and can be inserted directly into the microwave cavity. Interchangeable reservoirs allowed changing the relative humidity without disturbing the sample. The slides were rotated in the cavity so that the normal to the plane of the slide was either parallel ($\theta=0°$) or perpendicular ($\theta=90°$) to the external magnetic field. Randomly oriented samples were prepared in an analogous fashion using very fine glass beads supported on glass wool in place of the coverslip.

The ESR spectra were recorded on a Varian E-3 9.5 GHz spectrometer interfaced with a Varian 620/i 8K digital computer wth the scan speed controlled by an external oscillator. All spectra were recorded at room temperature (24–25 °C). Usual spectrometer settings were microwave power 5 mW, modulation amplitude 1 G, scan range 100 G, and filter time constant 0.1 to 1.0 s for scan speeds of 10–30 min. Digital processing and storage of data were performed as described earlier[15]. Spectral alignment was accomplished by the use of a dilute aqueous solution of di-*t*-butyl-nitroxide as an external standard.

Calculated spectra were generated on the University of Oregon time-sharing PDP-10 (Digital Equipment Corporation) and plotted on a Time Share Peripheral x–y recorder.

RESULTS AND DISCUSSION

Spectral analysis of isotropic samples

ESR spectra of spin-labeled membranous cytochrome oxidase are shown in Fig. 2. The samples are deliberately randomly oriented by coating small glass beads with the sample, in order to remove the complicating effects of partial orientation. The samples contain increasing amounts of phospholipid from top to bottom. Samples a–d contained 0.10, 0.24, 0.33, 0.49 mg phospholipid/mg protein, respectively. The samples were equilibrated over distilled water to achieve 100% relative humidity.

These spectra are easily characterized by the quantity $2A_m$, measured as the maximum splitting between the outermost lines (see distance between arrows, Fig. 2a). From Fig. 2, it can be seen that A_m remains almost unchanged as lipid content is increased from 0.10 to 0.24 mg phospholipid/mg protein, and then decreases monotonically with increasing lipid content. The decrease in A_m is a direct result of increasing molecular motion, and indicates increasing fluidity at higher phospholipid content.

It is difficult to tell by visual inspection of Fig. 2 whether there is a gradual change in one component or whether there are two distinct components present in different proportions. In our previous study[2] of cytochrome oxidase vesicles, labeled with 16-doxylstearic acid, two components were clearly identified. One component was immobilized and dominated the spectrum at low ratios of phospholipid/protein. The second, much more mobile component, increased with increasing phospholipid/protein ratios. By spectral subtraction and integration it was possible to estimate the

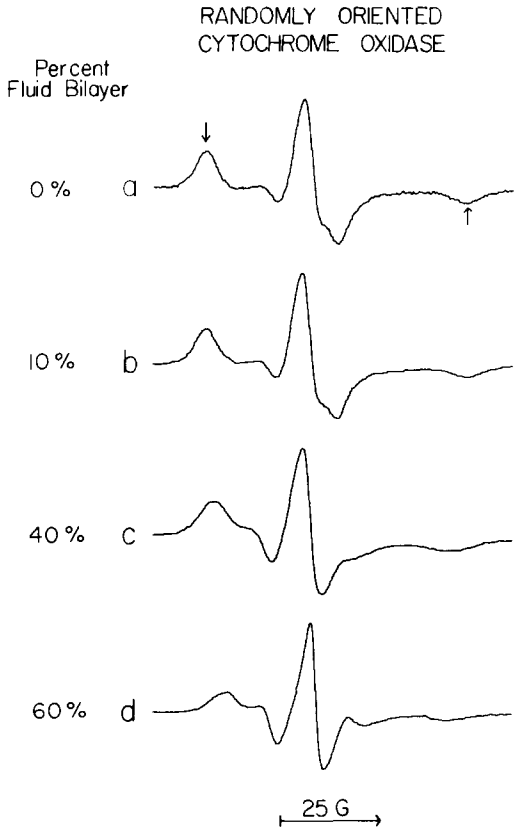


Fig. 2. ESR spectra of macroscopically disordered samples of cytochrome oxidase. The Spectra a–d are from samples that vary in lipid content. Expressed as mg phospholipid/mg protein (a) 0.10, (b) 0.24, (c) 0.33 and (d) 0.49. All samples are equilibrated at 100% relative humidity. Arrows (a) indicate the low and high field lines. Calculated proportions of fluid bilayer are shown at the left (see text).

percentages of bound components, and the results from those calculations[2] indicated that a relatively constant amount of the bound component was present regardless of the amount of the mobile component. Assuming the distribution of the lipid spin label accurately reflects the distribution of the phospholipids, the maximum amount of bound lipid is about 0.2 mg phospholipid/mg protein. When the lipid/protein ratio of the sample exceeds 0.2 mg phospholipid/mg protein, the additional phospholipid evidently forms fluid bilayer regions. The percentage of bilayer, calculated with the assumption that this constant value of bound phospholipid is present in each sample, is shown at the left of Fig. 2.

A similar set of calculations can be performed on the data of Fig. 2, but with larger uncertainties because of the greater similarity in the two spectral components. For example, Spectrum d of Fig. 2 (denoted by Σ_0) is represented by the linear combination

$$\Sigma_0 = \chi_b B_0 + \chi_f F_0 \tag{1}$$

where

$$\chi_b + \chi_f = 1 \tag{2}$$

and B_0 and F_0 represent the bound and fluid spectral components, respectively. B_0 is represented by Spectrum a of Fig. 2. This spectral component, B_0, is removed from Σ_0 by incremental subtraction of the digitalized data (spectral titration), yielding χ values consistent with those obtained earlier. For Spectra b, c, d of Fig. 2 χ_b is 0.9, 0.6, and 0.4, respectively. These may be compared with the two sets of corresponding values of 0.94, 0.65, 0.43 (spectral subtraction) and 0.95, 0.69, 0.34 (spectral addition) obtained using the 16-doxylstearic acid spin label[2,16].

Thus the amount of bound lipid estimated from these data is in agreement with the more accurate data obtained using the 16-doxylstearic acid. Based on this kind of spectral analysis, all of the spectra in Fig. 2 can be interpreted as consisting of two spectral components. These two components vary in relative proportions, accounting for the overall spectral changes observed in Fig. 2.

Response to hydration of isotropic samples

The parameter A_m varies with hydration as well as phospholipid content. The response to hydration for two cytochrome oxidase preparations and a control sample of cytochrome oxidase lipids are presented in Fig. 3. The response of the cytochrome oxidase lipids is similar to that observed previously for egg lecithin[14] and is characteristic of phospholipid bilayers.

It is interesting to note that the membranous cytochrome oxidase (0.49 mg phospholipid/mg protein) exhibits behavior similar to phospholipid bilayers. This is in contrast to the cytochrome oxidase preparation that has a much lower lipid content (0.10 mg phospholipid/mg protein). At low lipid to protein ratios, these preparations show almost no change in A_m over a wide range of hydration. These data suggest the presence of fluid bilayer regions in cytochrome oxidase preparations of higher lipid content. The absence of response to hydration at lower lipid levels is consistent

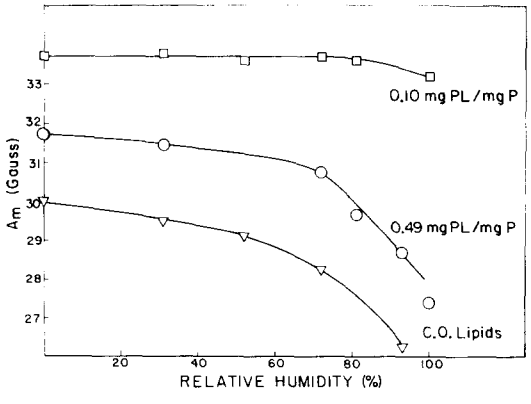


Fig. 3. Change in maximum splitting (A_m) as a function of relative humidity in the sample chamber. The three samples were randomly oriented cytochrome oxidase containing 0.10 mg phospholipid/mg protein (□—□), 0.49 mg phospholipid/mg protein (○—○) and cytochrome oxidase lipids (▽—▽). Note the similarity in response to hydration between the higher lipid content cytochrome oxidase and the isolated lipid fraction. PL, phospholipid; P, protein; C.O., cytochrome oxidase.

with the presence of a second environment at the protein–bilayer interface (boundary lipid), with properties unlike those of the fluid bilayer.

Macroscopically oriented cytochrome oxidase

Another criterion for phospholipid bilayer regions is the observation of anisotropy of oriented samples[17]. In our attempt to achieve macroscopic orientation, an aqueous suspension of cytochrome oxidase was allowed to settle onto glass slides and the excess aqueous phase removed. Orientation of these samples in the magnetic field gave the spectra shown in Fig. 4, where the solid lines are recorded with the magnetic

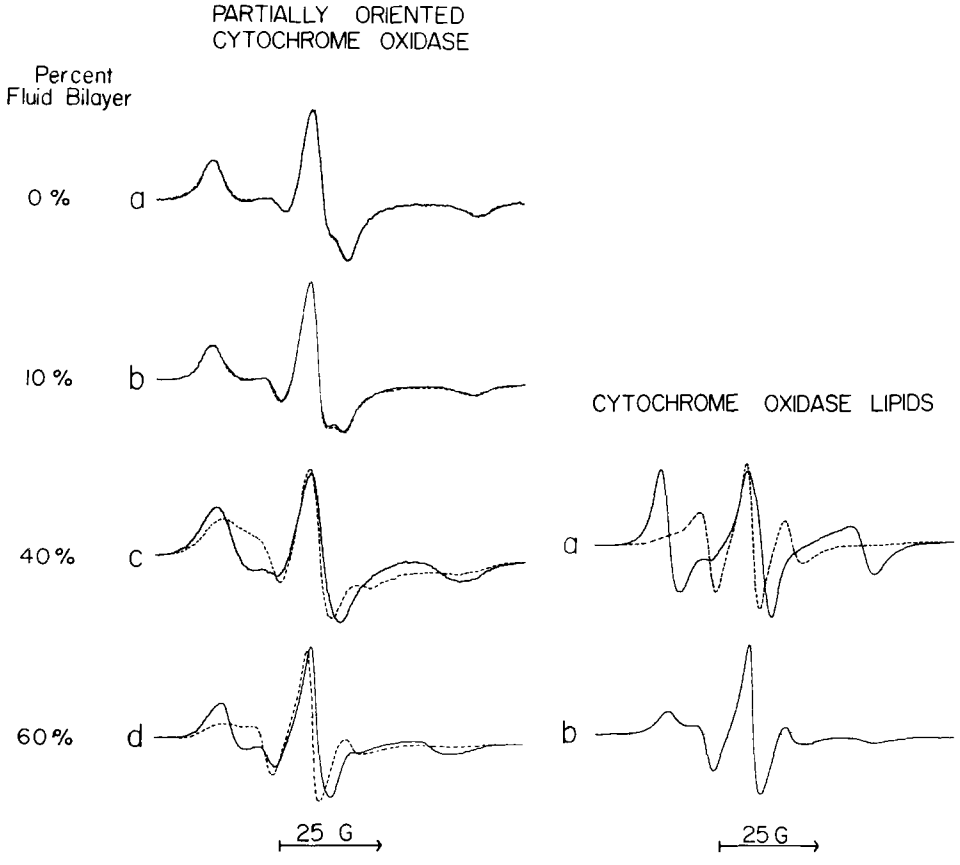


Fig. 4. ESR spectra of cytochrome oxidase oriented on a glass slide. The spectra were recorded with the magnetic field parallel (solid line) or perpendicular (dashed line) to the normal to the slide. The samples were at 100% relative humidity. The lipid content of the samples, in mg phospholipid/mg protein, was (a) 0.10, (b) 0.24, (c) 0.33 and (d) 0.49. (See Fig. 2 for the corresponding spectra of randomly oriented samples.) The anisotropy increases with increasing phospholipid content. Calculated proportions of fluid bilayer are shown at the left, assuming 0.2 mg boundary lipid/mg protein.

Fig. 5. ESR spectra of (a) ordered and (b) disordered samples of lipids extracted from membranous cytochrome oxidase and then spin labeled. In a the magnetic field is parallel (———) or perpendicular (----) to the normal to the slide. The sample is at 93% relative humidity, room temperature.

field along the normal to the plane of the sample. The dashed lines are the spectra recorded after the plane of the sample was rotated 90° in the magnetic field. The samples of Spectra a–d correspond to the randomly oriented samples of Fig. 2. In Fig. 4, there is essentially no anisotropy (macroscopic order) in the first sample (a) and only very slight anisotropy in Spectrum b. The anisotropy increases substantially as the phospholipid content increases. This is readily seen in Spectra c and d of Fig. 4.

The N–O moiety of the doxylstearic acid, containing the unpaired electron, is bonded to the flexible hydrocarbon backbone of the stearic acid by a fairly rigid spirane structure. As pointed out previously[17], this geometry dictates that the maximum splitting occurs with the magnetic field parallel to the long axis of the doxylstearic acid probe, and the minimum splitting with the magnetic field perpendicular to the long axis. In the spectra of Fig. 4 exhibiting anisotropy (*i.e.* Spectra c, d), the largest splitting occurs with the magnetic field normal to the plane of the sample and the smallest splitting is observed in the perpendicular direction. Thus, the data indicate that a fraction of the spin labels are oriented by intercalating into ordered bilayer regions. This ordered fraction increases with the phospholipid content of the cytochrome oxidase preparation, reflecting the increase in fluid bilayer.

Anisotropy has been observed previously in oriented samples of phospholipids[14,17–19] and some biological membranes[20]. The anisotropy usually observed in pure phospholipid bilayers is considerably greater than in preparations of biological membranes. The anisotropy exhibited by lipids extracted from cytochrome oxidase is shown in Fig. 5, along with the spectrum from the disordered sample. The anisotropy exhibited in Fig. 5a is marked, indicating a strong tendency for the lipids to orient with their long axes perpendicular to the glass support slide.

Spectral analysis of oriented samples

To complete this study it is of interest to examine the line shapes corresponding to ordered fluid bilayers in membranous cytochrome oxidase and in the extracted lipids. This information is contained in spectra of Figs 4d and 5a, but is partially obscured by overlapping spectral components. For example, the parallel and perpendicular spectra of Fig. 4d (denoted by $\Sigma_{\|}$, $\Sigma_{\perp}$) are evidently linear combinations of four components

$$\Sigma_{\|} = \chi_1 B_{\|} + \chi_2 B_0 + \chi_3 F_{\|} + \chi_4 F_0 \tag{3}$$

$$\Sigma_{\perp} = \chi_1 B_{\perp} + \chi_2 B_0 + \chi_3 F_{\perp} + \chi_4 F_0 \tag{4}$$

$$\chi_1 + \chi_2 + \chi_3 + \chi_4 = 1 \tag{5}$$

where B and F indicate normalized bound and fluid spectra, and the subscripts $_{\|,\perp}$, and $_0$ identify parallel, perpendicular and isotropic spectral components, respectively. The coefficients χ_1, χ_2, χ_3 and χ_4 are fractions of the total absorption contributed by the bound-ordered, bound-disordered, fluid-ordered, and fluid-disordered spectral components, respectively. The ordered components do not represent perfect orientation, but rather an ensemble of orientations described by a distribution function[17].

It is convenient to make the simplifying assumption that $\chi_1 = 0$, *i.e.*, that all bound lipid is disordered. This is consistent with the view that this lipid is tightly bound to an irregularly shaped protein complex. The line shapes B_0 and F_0 have

already been obtained from the isotropic samples (Fig. 2). Boundary lipid constitutes 0.2 mg phospholipid/mg protein[2], hence the fraction of bound lipid in Spectrum d (Fig. 2) is $\chi_2 = 0.2$ mg/0.49 mg $= 0.41$, and B_0 is removed from Eqn 3 by one digital subtraction. The remaining isotropic component, F_0, is removed by spectral titration yielding χ_4, χ_3 and $F_{\parallel}$ component. This procedure was repeated on the perpendicular spectrum using Eqn 4 as a basis for analysis. The overall accuracy is limited by the assumptions outlined above and by the difficulty in obtaining precise end points in spectral subtractions of components having very similar line shapes. The final set of χ values were, however, consistent for the two orientations (Eqns 3 and 4). The numerical values based on Spectrum d of Fig. 4 are $\chi_1 = 0$, $\chi_2 = 0.41$, $\chi_3 = 0.26$ and $\chi_4 = 0.33$. The χ_3 is a measure of the fraction of macroscopically ordered fluid bilayer, and therefore approximately one quarter of the spin labels are oriented with respect to the external magnetic field. This number represents a lower limit to the microscopic order of the fluid bilayer since partial macroscopic disorder on the glass slide will not have been subtracted out. The final line shapes F_0, $F_{\parallel}$ and $F_{\perp}$ are plotted in the left column of Fig. 6.

Obtaining the control spectra from the extracted lipids is substantially simpler because of the absence of the bound component. Defining the parallel, perpendicular and isotropic spectra as $\Sigma'_{\parallel}$, $\Sigma'_{\perp}$ and Σ'_0, respectively, the appropriate relations are

$$\Sigma'_{\parallel} = \chi'_3 F'_{\parallel} + \chi'_4 F'_0 \tag{6}$$

$$\Sigma'_{\perp} = \chi'_3 F'_{\perp} + \chi'_4 F'_0 \tag{7}$$

$$\Sigma'_0 = F'_0 \tag{8}$$

$$\chi'_3 + \chi'_4 = 1 \tag{9}$$

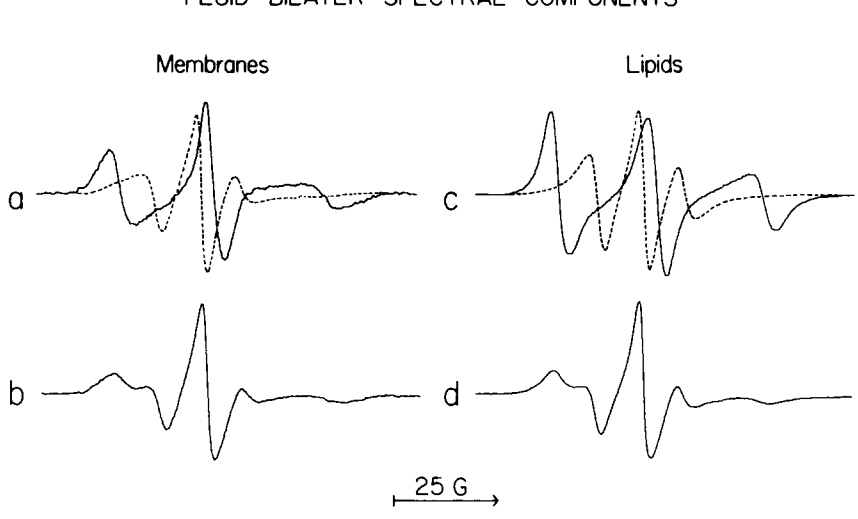


Fig. 6. Spectral components (difference spectra) resulting from a series of subtractions using digitalized data (see text). In the top row are spectra of the oriented fluid bilayer components (parallel, solid line; perpendicular, dashed line, as in Figs 4 and 5). In the bottom row are fluid bilayer spectral components of the corresponding disordered samples. These isolated spectral components permit a comparison of fluid bilayer regions in membranous cytochrome oxidase with the lipid samples.

where primes denote the pure lipid sample, and the other quantities are defined as above. The results of the spectral analysis on spectra of Fig. 5 are $\chi'_3 = 0.82$ and $\chi'_4 = 0.18$. Thus, approximately three quarters of the lipid spin labels are macroscopically ordered with respect to the glass slide.

The line shapes F'_0, $F'_{\parallel}$ and $F'_{\perp}$ obtained for the oriented lipids are plotted in the right column of Fig. 6. These line shapes are remarkably similar to F_0, $F_{\parallel}$ and $F_{\perp}$ (left column Fig. 6) of the cytochrome oxidase membranes. This confirms the presence of ordered fluid bilayer regions in membranous cytochrome oxidase.

A more quantitative comparison between the fluid bilayer components can be made using the line shape calculations developed earlier[14,17]. In this approach a visual fit of the experimental spectral components is made, using a computer program based on a Gaussian distribution of orientations and a restricted amplitude of anisotropic molecular motion. The results of these calculations (Fig. 7) provide a good fit to the experimental spectral components (Fig. 6). The primary parameters varied to achieve this fit are the molecular motion parameter, γ, and the Gaussian distribution parameter, θ_0 (increasing γ and θ_0 values indicate increasing molecular motion and decreasing order, respectively). Spectral components b and d (Fig. 6) were simulated by setting $\theta_0 = 10000$ (essentially random orientation) and varying γ. The resulting values of γ were then fixed, and θ_0 was varied to simulate the oriented spectral components a and c (Fig. 6). The results of these calculations (Fig. 7) indicate the molecular

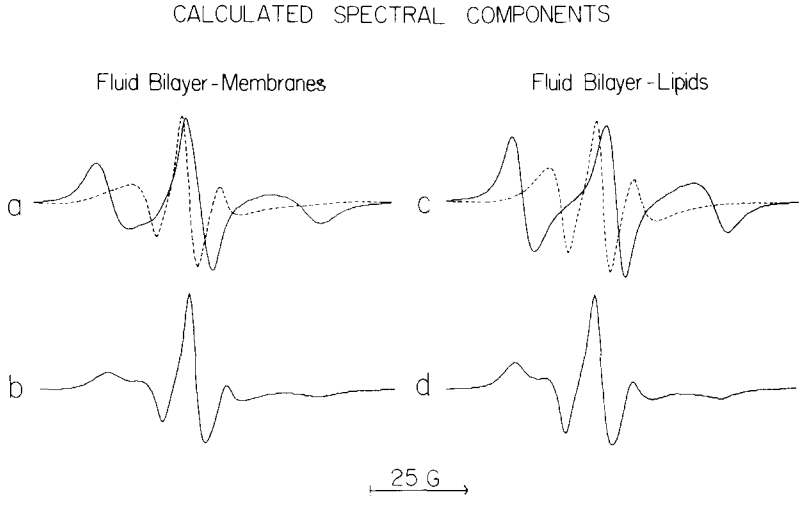


Fig. 7. Fluid bilayer spectral components calculated assuming a distribution of orientations and anisotropic molecular motion. Spectra a–d are computer simulations of the corresponding spectral components of Fig. 6. The input parameters are $A_{xx} = 5.9$ G, $A_{yy} = 5.4$ G, $A_{zz} = 34.0$ G, $g_{xx} = 2.0088$, $g_{yy} = 2.0058$, $g_{zz} = 2.0022$. These simulations are for a microwave frequency of 9.5 GHz and are based on a set of 2000 molecules. The spectra in the left column had a line width of $w = 3.2$ G, $w' = 0.7$ G; in the right column $w = 3.2$ G, $w' = 0.4$ G, where the line width function used was $w + w'$ $(3\cos^2\theta - 1)$, and θ is the angle between the magnetic field and the normal to the plane of the bilayer. The line shape chosen was Lorentzian; the relative line heights were 0.7:1.0:0.5 (left column) and 0.95:1.0:0.55 (right column). The motion parameter, γ, is 41.5° (left column), and 45.0° (right column). The orientation distribution parameter, θ_0, for both components of a is 65 and for both components of c is 45. For Spectra b and d, $\theta_0 = 10000$. (See text and refs 14 and 17.)

motion of the fluid components of the samples is very similar (γ is 41.5° for the membranes and 45.0° for the isolated lipids, respectively), with only slightly more hindered motion present in the membrane sample. The order of the fluid component of the membrane sample is less than that of the lipids (θ_0 is 65 for the membranes and 45 for the lipids). Thus the small differences in line shapes (Fig. 6) are attributable to a slightly lower degree of molecular motion and a broader distribution of orientations in the membranous cytochrome oxidase fluid bilayer regions compared to the control where protein is absent.

The data from this study are consistent with the structure shown in the cross-sectional diagram of Fig. 8. The dimensions and several features of this model are from Vanderkooi *et al.*[21]. The hydrophobic surface (lightly cross-hatched) of the amphipathic protein complex is coated by an immobilized boundary lipid layer (densely cross-hatched). The orientation of the boundary lipid is deliberately obscured, although the orientation of the bound lipid chains is assumed to be random. The hydrophobic binding surfaces of the protein complex must be microscopically irregular, in order to form the potential wells responsible for the boundary lipid immobilization and calculations based on this assumption yield plausible line shapes. Protein–protein bridges are not shown in this simplified model.

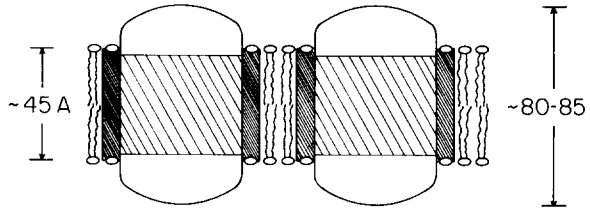


Fig. 8. A cross-sectional view of the cytochrome oxidase membranes as deduced from the available data. The core of the membrane is composed of hydrophobic parts of the cytochrome oxidase complex (lightly cross-hatched) surrounded by an immobilized boundary layer of lipid (densely cross-hatched) intercalated into fluid lipid bilayer.

Beyond the boundary layer is a more fluid phospholipid bilayer region. This fluid bilayer exhibits the orientation and response to hydration characteristic of bilayers of phospholipids. The bilayer regions are restricted in extent, but the spectral features are remarkably similar to those observed in the extensive fluid bilayers formed from the isolated lipids. Evidently, the lipid–protein interaction decreases very rapidly with distance in the bilayer plane. These short-range interactions may produce second order effects on lipids outside of the boundary layer, but the essential features are those of classical bilayer.

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