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PROTEIN - IMMOBILIZED LIPID IN DIMYRISTOYLPHOSPHATIDYLCHOLINE -SUBSTITUTED CYTOCHROME OXIDASE: EVIDENCE FOR BOTH BOUNDARY AND TRAPPED-BILAYER LIPID.

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SUMMARY
Cytochrome oxidase-dimyristoyl phosphatidylcholine complexes have been prepared at defined lipid:protein ratios to study the effects of protein packing density on the lipid fluidity. All the complexes reveal a two-component ESR spectrum from an incorporated phosphatidylcholine spin label, corresponding to both an immobilized lipid boundary layer and fluid bilayer regions. Difference spectra, obtained by subtracting the same immobilized spectrum from the spectra of the various complexes, demonstrate a strong perturbation of the lipid bilayer fluidity which is quite distinct from the immobilized boundary layer formation.

Lipid-protein interactions are almost certainly important determinants of biological membrane structure and function. The enzymatic and transport properties of membrane proteins have been shown to be sensitive both to the membrane lipid composition and to the fluidity of the lipid chains (1,2,3). Since the lipid bilayer is the major permeability barrier of the membrane, perturbation of this structure by the presence of membrane proteins may also determine membrane properties. It is this latter aspect which we have investigated.

Previous workers have provided evidence for specific immobilization of a boundary layer of lipid in immediate contact with integral membrane proteins (4). These effects are most evident at low lipid:protein ratios , and it has been suggested by other workers (5) that, at least in certain instances, the immobilization may not correspond to a specific boundary layer but consist of regions of very strongly perturbed bilayer, trapped between the protein molecules. We have investigated a particular membrane protein, cytochrome oxidase, with a single lipid environment, dimyristoyl phosphatidylcholine, in an attempt to distinguish between these effects. The

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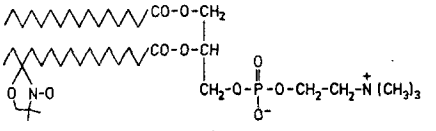
results demonstrate the coexistence of both strongly-immobilized boundary lipid and bilayer lipid at all lipid:protein ratios studied. At lower lipid:protein ratios the bilayer becomes progressively more strongly perturbed, indicating motional restriction of the entrapped bilayer regions which is quite distinct from the boundary layer immobilization.

MATERIALS AND METHODS

Dimyristoyl phosphatidylcholine was obtained from Fluka, (Buchs, Switzerland) and ran as a single spot in thin layer chromatograms. Cholic acid (Sigma, Munich, W.Germany) was recrystallized from ethanol-water (2:1) and then converted to the sodium salt. The phosphatidylcholine spin-label I(3,12) was prepared according to the general methods of Hubbell and McConnell (6). Cytochrome oxidase was purified from yeast according to the method of Eytan and Schatz (7), and had comparable homogeneity and specific activity to that reported by these authors. The buffer used throughout this study was tris (10 mM, pH 7.0), KCl (1.0 M),sucrose (1%).



I (3,12)

Replacement of the endogeneous lipid and detergent in the cytochrome oxidase preparation was effected by three exchanges for dimyristoyl phosphatidylcholine in the presence of 0.2% sodium cholate, essentially according to the method of Warren et.al. (8). Samples with different lipid:protein ratios were prepared by one of two procedures. For complexes with lipid:protein ratios less than 100:1, the twice-exchanged enzyme was incubated with dimyristoyl phosphatidylcholine in the presence of different cholate concentrations (0.2 - 0.5%) followed by ammonium sulphate precipitation. For complexes with lipid:protein ratios greater than 100:1, the twice-exchanged enyzme was incubated with appropriate amounts of dimyristoyl phosphatidylcholine in the presence of 1.25% cholate, according to the procedure of Hesketh et.al. (3). The phosphatidylcholine spin label I(3,12) was present in these last lipid exchange media at a concentration of 2 mole% relative to the dimyristoyl phosphatidylcholine. Cholate was removed from the sample by dialysis against buffer containing Amberlite XAD2 (B.D.H., Poole, England) for 15h. at 15°C. ^{14}C-cholate assays showed that under these conditions the residual cholate is reduced to less than 3 moles/mole of enzyme. Lipid phosphate concentration was measured according to Eibl and Lands (9), and protein concentration according to Dully and Grieve (10).

ESR spectra were recorded on a Varian E-12 9GHz spectrometer equipped with nitrogen, gas-flow, temperature regulation system. Spectra were digitized on paper tape (1K points/100 gauss) using a Hewlett Packard 3450B/2547A/2753A data collection system, and processed on a PDP11/34 computer with Textronix 4006 display.

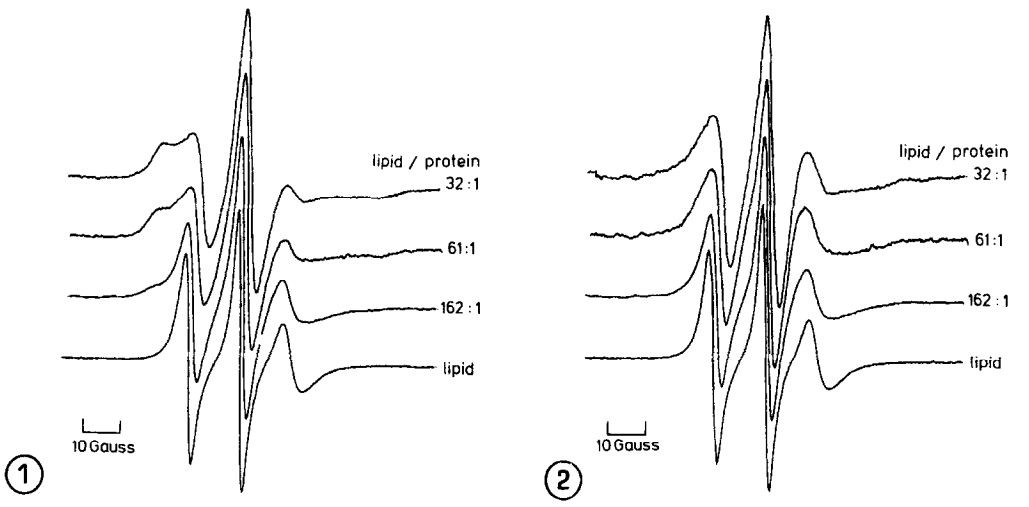


Fig.1. ESR spectra of phospholipid spin label I(3,12) in cytochrome oxidase-dimyristoyl phosphatidycholine complexes at various mole ratios. T=28°C; right-hand column gives the phospholipid:protein mole ratio in the complex.

Fig.2. Lipid bilayer components of the ESR difference spectra of cytochrome oxidase-dimyristoyl phosphatidylcholine complexes. T=28°C; right-hand column gives the phospholipid:protein mole ratio in the complex.

RESULTS

The ESR spectra of spin label I(3,12) in the various cytochrome oxidase-dimyristoyl phosphatidylcholine complexes at 28°C (Fig.1) all display two components, the ratio of the fluid to the strongly immobilized component becoming greater with higher lipid:protein ratio (c.f. ref.11). At this temperature the samples are above the ordered-fluid phase transition (23°) of dimyristoyl phasphatidyl-choline bilayers, as is clearly demonstrated by the spectrum of the lipid alone.

The two-component nature of the spectra is clearly revealed in the difference spectra of Fig.2 which were obtained by subtracting the same immobilized-component spectrum from the spectra of each of the different lipid:protein ratios. The immobilized component spectrum was obtained from an extensively delipidated complex in which no bilayer component was evident and which best matched the spectral splittings of the immobilized component in the complexes. The resulting difference spectra of Fig. 2 are characteristic of the spin label I(3,12) in fluid lipid bilayers where the amplitude

Table 1. Motional parameters of spin label I(3,12) in lipid bilayer regions of cytochrome oxidase-dimyristoyl phosphatidylcholine complexes as a function of lipid:protein ratio. (S, order parameter; a_o, isotropic hyperfine constant; γ_{max}, motional amplitude).

Lipid/protein (mole/mole)	S	a_o(gauss)	γ_{max}(deg)
32:1[a]	0.49 - 0.34	14.5-13.8	52^o - 62^o
61:1	0.28_2	13.6	66^o
162:1	0.17_9	13.7	74^o
lipid	0.11_3	14.2	79^o

[a]spectra indicate 2 or more bilayer environments

of motion of the label is large (11). At high lipid:protein ratio the spectra correspond fairly closely to those of bilayers of dimyristoyl phosphatidylcholine alone, but at decreasing lipid:protein ratio the spectra become broader and the spectral anisotropy increases indicating a decreased amplitude (and possibly also rate) of motion of the bilayer lipid chains. These changes have been quantitated by analysis of the spectral splittings to yield the order parameters ,isotropic hyperfine constants and effective angular amplitudes of restricted random walk motion (11) given in Table 1. This clearly demonstrates the progressive immobilization of the lipid chains in the bilayer regions as the protein packing density increases.

DISCUSSION

Similar two-component spectra to those of Fig.1 have been observed by Jost et.al. (4) in progressively lipid-extracted samples of beef-heart cytochrome oxidase. From the lipid/protein titration, these latter authors concluded that the strongly-immobilized component corresponded to a single, boundary-layer shell of lipid molecules surrounding the protein. Quantitation of the relative amounts of the two components in the present system (12),and the finding that the immobilized component at all the lipid:protein ratios in Fig. 1 can be represented by the same spectrum, are both consistent with a similar boundary-lipid interpretation.

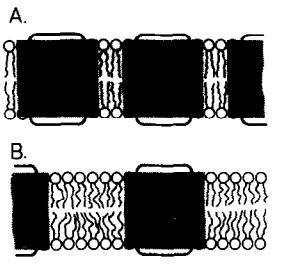


Fig.3. Schematic indication of the molecular packing in cytochrome oxidase-dimyristoyl phosphatidylcholine complexes. A.: at low lipid:protein mole ratio; boundary lipid and perturbed (less fluid) lipid bilayer. B.:at high lipid:protein mole ratio; boundary lipid and unperturbed (more fluid)lipid bilayer.

The results of Figs.1 and 2 thus demonstrate a clear distinction in this system between lipid boundary-layer immobilization and bilayer perturbation by the protein packing. As illustrated in Fig.3, at high protein packing density both effects are operative, but at low protein density only boundary-layer immobilization is present with little or no perturbation of the bilayer regions. This may not be the case in all systems:Chapman et.al.(5) have concluded that the immobilized lipid component,which is only observed at lipid:protein ratios approaching equimolar,in gramicidin A-lipid complexes corresponds solely to lipid entrapped in polypeptide clusters. However gramicidin A is a much smaller molecule and presumably has somewhat different hydrophobic faces than cytochrome oxidase and thus may not have the structural features required for boundary layer formation. It seems likely that the results with cytochrome oxidase are more appropriate to the situation of large integral proteins in biological membranes and those with gramicidin A to that of small integral proteins, e.g. monomeric glycophorin. Thus it is clear that protein packing density can have considerable influence on the intervening lipid bilayer regions and hence on those membrane properties which are sensitive to bilayer fluidity. It is interesting to note that the lipid:protein ratio in the inner mitochondrial membrane lies in the range for which strong perturbation of the lipid bilayer is observed in Fig. 2, suggesting that such effects may be important in the natural situation.

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