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Cytochrome *c* Induced Lateral Phase Separation in a Diphosphatidylglycerol–Steroid Spin-Label Model Membrane†

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ABSTRACT: The extrinsic membrane protein cytochrome *c* binds to lipid mixtures containing negatively charged phospholipids such as diphosphatidylglycerol (DPG). In this study the effect of cytochrome *c* on the lipid distribution in a DPG–steroid spin-label (3-doxyl-5α-cholestane) model membrane system is examined. The electron spin resonance (ESR) line-shape changes indicate that cytochrome *c* induces lateral phase separation at room temperature. The resulting two-dimensional lipid distribution is nonrandom, consisting of clusters of phospholipids bound to cytochrome *c* and patches of steroid spin-label molecules. Phase separations are also observed in the three-component system: DPG, phosphatidylcholine, and 3-doxyl-5α-cholestane.

Lipid-protein interactions in biological membranes can be examined by observing differences in mobility of lipid spin-labels (Griffith and Jost, 1976). In the large protein complex cytochrome oxidase (cytochrome a, a_3), the terminal member of the mitochondrial electron transport chain, a fraction of the lipid is observed to be immobilized (Jost et al., 1973). This is consistent with the idea that cytochrome oxidase and other integral proteins penetrate deeply into or completely through the phospholipid bilayer, creating a hydrophobic lipid–protein interface. In contrast to the behavior of integral proteins, extrinsic proteins such as cytochrome *c* are easily released from membranes by dilute salt solutions (Jacobs and Sanadi, 1960). In its normal functional role, the small protein cytochrome *c* transfers electrons from one large integral protein complex (cytochrome b–c_1) to another (cytochrome oxidase), but it is also known to bind to bilayers containing negatively charged lipids (Green and Fleischer, 1963; Gulik-Krzywicki et al., 1969; Nicholls, 1974; Kimelberg et al., 1970; Vanderkooi et al., 1973), and specific lipid–protein interactions may be important to the function of cytochrome *c*. In a previous study, the motion of fatty acid spin-labels before and after addition of cytochrome *c* was examined in bilayers of the negatively charged phospholipid diphosphatidylglycerol (DPG, cardiolipin) and phosphatidylcholine (PC, lecithin).[1] Any effects of cytochrome *c* were small compared to the marked immobilization by integral proteins (Van and Griffith, 1975). These data are consistent with electrostatic binding of cytochrome *c*.

In this paper we examine whether cytochrome *c* can influence the local arrangement of lipids in the plane of the mem-

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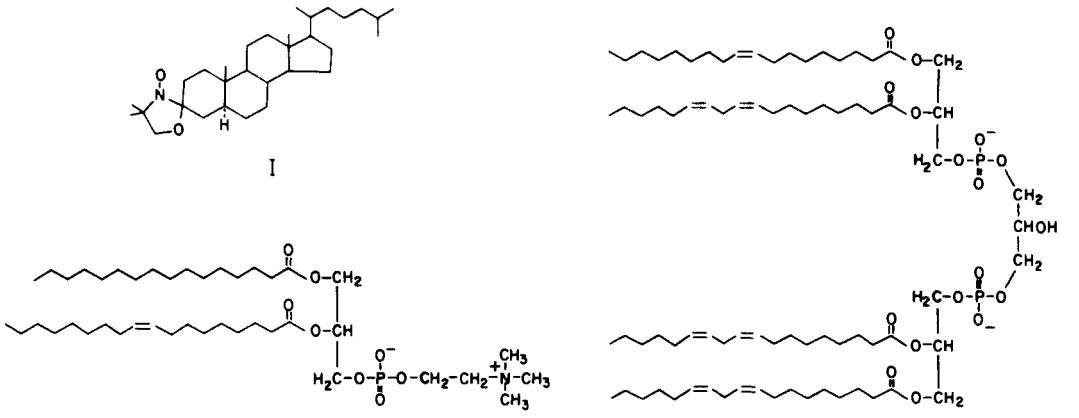
[1] Abbreviations used are: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; ESR, electron spin resonance.



I

II

III

FIGURE 1: Structures of 3-doxyl-5α-cholestane spin-label (I), phosphatidylcholine (II), and diphosphatidylglycerol (III). The lipid side chains shown in II and III are major components but there is some heterogeneity resulting from variations in degree of unsaturation and chain length in the naturally occurring phospholipids used in these experiments.

brane, an effect that might not show up in the segmental motion of the lipids. The membrane model system chosen is a mixture of the lipids shown in Figure 1: the steroid spin-label 3-doxyl-5α-cholestane in DPG and in DPG–PC mixtures. The experimental design is influenced by the interesting studies of Träuble and Sackmann (1972) on lipid distributions in the steroid spin-label–PC model system and of Ohnishi and Ito (1973, 1974) on Ca^{2+}-induced phase separations in lipid bilayers.

Materials and Methods

Materials

Cytochrome *c* (type VI from horse heart) and DPG (beef heart) were purchased from Sigma Chemical Company and were used without further purification. Egg yolk PC was isolated and purified according to the method of Pangborn (1941, 1951). The steroid spin-label, 3-doxyl-5α-cholestane, was synthesized from 5α-cholestan-3-one (Keana et al., 1967; Marriott et al., 1975) and 5-doxylstearic acid was purchased from Syva. The term doxyl refers to the 4′,4′-dimethyloxazolidinyl-*N*-oxy derivative of the corresponding ketone (see Figure 1).

Methods

DPG–Egg PC–Cytochrome c Complexes. DPG (1.0 mg) and egg PC (1.0 mg), both dissolved in ethanol, were added to a small vial along with 24 μl of a stock solution (5 mg/ml in chloroform) of 3-doxyl-5α-cholestane. The solvent was evaporated under nitrogen gas and the vial containing the lipids was placed under vacuum for 1 h to remove any remaining solvent. The lipids were then resuspended by vortexing in 0.5 ml of 0.02 M Tris-HCl (pH 8.5) containing 6 mg of cytochrome *c*. Resuspension of the lipids resulted in the formation of a water-insoluble lipid–cytochrome *c* complex. The turbid solution was centrifuged at 3000*g* for 10 min at 4 °C, and the supernatant containing excess cytochrome *c* was discarded. The red precipitate was redispersed in 0.5 ml of 0.02 M Tris-HCl (pH 8.5) and recentrifuged. The redispersion–centrifugation procedure was then repeated. The pellet from the last centrifugation was used for the electron spin resonance (ESR) measurements.

DPG–Cytochrome c Complexes. One milligram of DPG, dissolved in ethanol, and 12 μl of the stock spin-label solution were dried down under nitrogen. After pumping to remove the solvent, the spin-label–DPG mixture was resuspended in 0.4 ml of 0.02 M Tris-HCl (pH 8.5) containing 3 mg of cytochrome *c* by bath sonication (0 °C). Unlike the DPG–PC–cytochrome *c* complex, the DPG–cytochrome *c* complex is water soluble, and no attempt was made to remove the excess cytochrome *c* in this case.

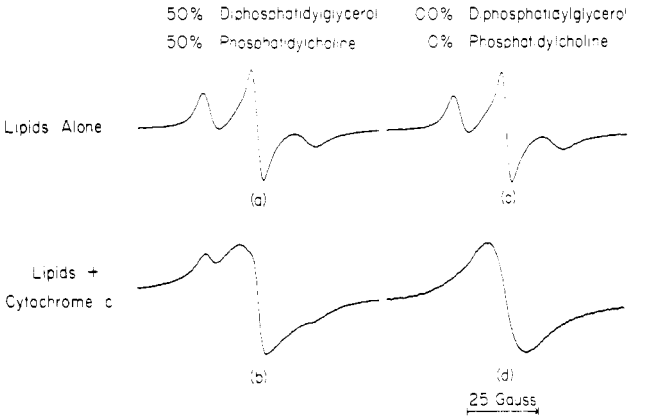


FIGURE 2: ESR spectra of 3-doxyl-5α-cholestane in isotropic samples of lipid alone and lipid–cytochrome *c* complexes. The spectra were digitalized with a Varian 620/L computer and replotted to the same vertical scale.

ESR Spectra. ESR spectra were recorded at 24 °C on a Varian E-Line 9.5-GHz ESR spectrometer.

Results

The Steroid Spin-Label in DPG and DPG–PC Aqueous Dispersions. ESR spectra of the dry lipids dispersed in aqueous buffer are shown in the top row of Figure 2. These spectral line shapes are typical of the steroid spin-label in bilayers of phospholipids. Some exchange broadening is present because of the relatively high spin-label concentration of 1:20 molar ratio of spin-label to phospholipid side chains (e.g., 1:10 spin-label to PC or the equivalent of 1:5 spin-label to DPG which has four lipid side chains). The spectra in the bottom row of Figure 2 are observed when the same lipid films are taken up in aqueous buffer containing cytochrome *c*. The effects of cytochrome *c* on a lipid mixture 1:4 DPG:PC are not as striking, but are clearly evident. No effect is observed in PC bilayers without DPG present.

These large effects of cytochrome *c* can be observed under a variety of experimental conditions. For example, omission of the centrifugation step (i.e., removal of excess cytochrome *c*) in the 1:1 DPG:PC preparation yields a spectrum indistinguishable from the corresponding one of Figure 2. Experiments on the 1:0 DPG:PC lipid sample (0.02 M Tris-HCl, pH 8.5) were repeated in 0.1 M phosphate (pH 7.0), and the spectra were again the same as the corresponding spectra of Figure 2.

The data of Figure 2 were obtained using cytochrome *c* as received from the supplier. By comparing the ratio of the optical absorbances of this sample at 548.4 and 360 nm with those of the completely oxidized ($K_3Fe(CN)_6$ treated) and reduced ($Na_2S_2O_4$ treated) forms (Margoliash and Frohwirt, 1959), more than 95% of the cytochrome *c* was found to be in the oxidized (Fe^{3+}) state. Experiments were also performed with cytochrome *c* treated with $Na_2S_2O_4$ and chromatographed on Sephadex G-25 (Andréasson et al., 1972). This sample was predominantly (>90%) in the reduced form. In these experiments spectral line-shape changes similar to those of Figure 2 were observed.

PC Spin-Label in Aqueous Dispersions of DPG. Another variation of the experimental procedure would be to substitute a PC spin-label for the steroid spin-label I. We have performed experiments with PC substituted at the β position with either 12-doxylstearic acid or 16-doxylstearic acid. The molar lipid ratio was 1:5 spin-labeled PC:DPG. Unfortunately, a significant destruction of the ESR signals of these PC spin-labels occurs when both DPG and cytochrome *c* are present. One sample of DPG was hydrogenated (10% Pd on carbon in ethanol), and the experiment was repeated using the PC derived from 16-doxylstearic acid. The destruction of the spin-label in this case is greatly reduced. Addition of cytochrome *c* produces at most only small line-shape changes, in contrast to the steroid spin-label results described above. We find no convincing evidence from these exploratory experiments that cytochrome *c* induces phase separations in the two-component system, DPG and spin-labeled PC.

Fatty Acid Spin-Label in Aqueous Dispersions of DPG. Fatty acids can act as detergents, so that it is questionable whether fatty acid spin-labels are useful at the high label concentrations required in phase separation studies. However, at low concentrations these spin-labels can be used to measure motion before and after the addition of cytochrome *c* (Van and Griffith, 1975). We have diffused 5-doxylstearic acid into the 1:4, 1:1, and 1:0 by weight DPG:PC samples (spin-label:total lipid mole ratio = 1:100). The order parameters (determined using Method II, Griffith and Jost, 1976) measured from the room temperature ESR spectra of the first two of these samples are 0.55 (0.57) and 0.55 (0.58), respectively, with all data = 0.02. The numbers in parentheses are for the corresponding samples after the addition of cytochrome *c*. There is some destruction of signal in all three samples, and signal destruction in the pure DPG (1:0) sample was rapid so no order parameter was obtained for this sample. Signal destruction is greatly reduced when hydrogenated DPG is used. The order parameters for 1:4, 1:1, and 1:0 by weight hydrogenated DPG:PC are 0.56 (0.58), 0.55 (0.59), and 0.49 (0.67), respectively.

Discussion

Cytochrome *c* clearly has a pronounced effect on the ESR spectra of the steroid spin-label intercalated into lipid mixtures containing DPG, as shown in Figure 2. The resulting line shape is dominated by spin exchange, indicating clustering of the steroid spin-labels. Transitions from three line spectra to one single exchange-narrowed line are well known in progressively more concentrated solutions of spin-labels (Wertz and Bolton, 1972; Jost and Griffith, 1972) and these line shapes have been treated in detail in lipid phase transition and lateral diffusion studies (Sackmann and Träuble, 1972; Devaux et al., 1973). These line shapes have also been reported in a number of interesting studies of lipid phase separations induced by Ca^{2+} in phosphatidic acid–PC and phosphatidylserine–PC bilayers (Ohnishi and Ito, 1973, 1974; Ito and Ohnishi, 1974; Galla and Sackmann, 1975), and by Ca^{2+} and polylysine in phosphatidic acid–PC bilayers (Galla and Sackmann, 1975).

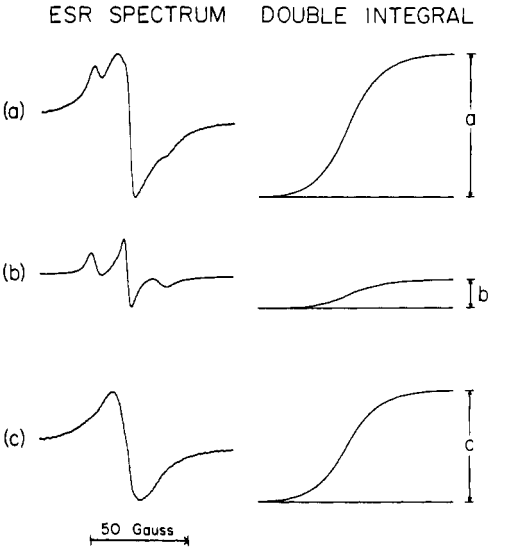


FIGURE 3: First derivative spectra of the steroid spin-label in 1:1 mixtures of DPG and egg PC: (a) DPG + PC + cytochrome *c*; (b) DPG + PC; (c) the result of a spectral subtraction of spectrum b from a in which 22.5% of the total absorption was subtracted. The first derivative spectra and double integrals of b and c are scaled to reflect the concentrations of the two components present in the composite spectrum a. The heights of the double integrals are $a = 9.53$, $b = 2.14$, and $c = 7.39$ in arbitrary units.

The single line spectrum d of Figure 2 resembles the spectrum of pure spin-label, indicating a high degree of lateral phase separation. As the ratio of DPG:PC is decreased to 1:1, the phase separation of the spin-label is still very apparent. Further decreasing of the ratio to 1:4 substantially reduces the phase separation and the effect disappears completely in bilayers without DPG. In order to determine whether line shape b of Figure 2 is compatible with two spin-label environments present at the same time, spectral titrations were performed by subtracting incremental amounts of the lipids-alone spectrum a from b until an obvious end point was reached (e.g., inverted peaks). The results are given in Figure 3. The difference spectrum at the end point (Figure 3c) represents about 80% of the total absorption, and is a typical exchange narrowed line shape remarkably similar to Figure 2d. The subtraction procedure was repeated using a spectrum similar to Figure 3b, except at much lower spin-label concentration (1:100 spin-label:lipid instead of 1:10) with similar results. In this case the difference spectrum represents approximately 95% of the total absorption but the final line shape was slightly distorted. We conclude that cytochrome *c* is interacting with lipids on both sides of the bilayers in this model system since the spin-label is almost certainly distributed on both sides and 80–95% of the spin-label is clustered after addition of cytochrome *c*, rather than a maximum of 50% expected if cytochrome *c* were present only on the outside of the lipid vesicles.

Cytochrome *c* evidently does not penetrate deeply into these

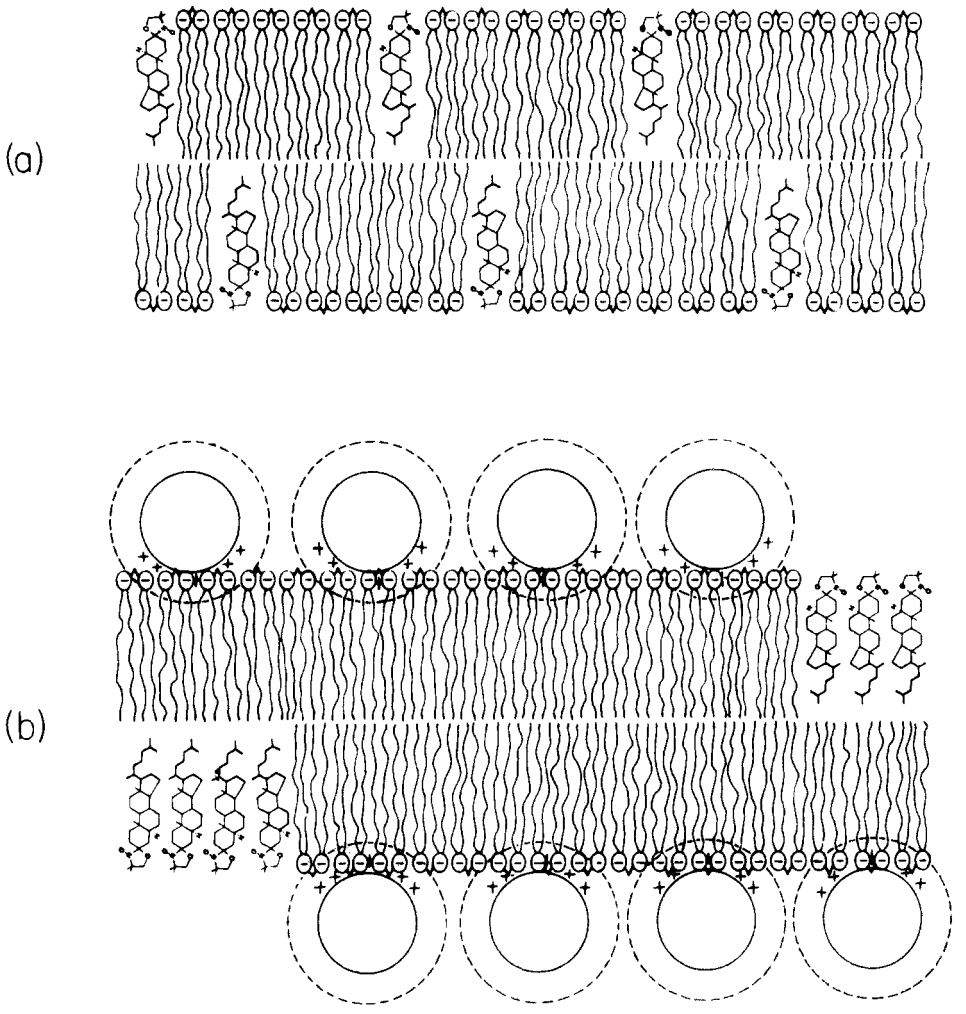


FIGURE 4: Schematic diagram indicating the distribution of lipids in the absence (a) and in the presence (b) of cytochrome *c*.

lipid bilayers. The segmental motion at the 5 position of the fatty acid spin-label, as indicated by the order parameter, is essentially unchanged by cytochrome *c* in the 1:4 by weight DPG:PG bilayers. This is consistent with a previous study of molecular motion in 1:4 DPG:PC bilayers (Van and Griffith, 1975). As the percent of DPG in the lipid mixture is increased, we do observe an effect of cytochrome *c* on motion. These effects are, however, small compared to the large reduction in lipid chain motion observed when intrinsic proteins known to penetrate deeply into or completely through the phospholipid bilayers are present (e.g., cytochrome oxidase; Jost et al., 1973).

The essential results are summarized in the diagrammatic sketches of Figure 4. Figure 4a depicts a cross-sectional view of the lipid bilayer containing the steroid spin-label. The distribution is predominantly random, although some clustering may be present. Figure 4b shows the probable distribution after addition of cytochrome *c*. The multiple positive charges on each cytochrome *c* (Dickerson, 1972) attract the negatively charged DPG lipids. As a result, patches of spin-labels are formed by exclusion as shown in Figure 4b. In mixtures of DPG and PC the same effect is observed, but the effect diminishes with decreasing DPG content. The interactions are electrostatic and the cytochrome *c* does not penetrate deeply into the phospholipid bilayers, as judged by the fatty acid spin-label data.

The common phospholipids which carry net negative charges near neutral pH include DPG, phosphatidic acid, phosphatidylserine, and phosphatidylinositol. We have repeated the experiments of Figure 2 replacing DPG with each of the remaining three negatively charged lipids. The ESR spectra of the steroid spin-label dissolved in the lipids alone are similar to those of Figure 2. Cytochrome *c*, however, has a much smaller effect on the phosphatidic acid than in DPG, and very little if any effect on the phosphatidylserine and phosphatidylinositol, or the mixtures of these lipids (1:1) with phosphatidylcholine. It is interesting to compare these results with recent literature data on Ca^{2+}-induced phase separations in mixtures of these negatively charged lipids containing 1-20% spin-labeled phosphatidylcholine. Ca^{2+} induces a lipid phase separation in spin-labeled PC mixtures with phosphatidylserine and phosphatidic acid, but not with either DPG or phosphatidylinositol (Ohnishi and Ito, 1974; Galla and Sackmann, 1975). Thus, there is evidence for specificity in the tendency for lateral phase separation and the specificity is not the same for cytochrome *c* and Ca^{2+}. Molecular models suggest that one Ca^{2+} ion fits between the two phosphates of the DPG molecule, minimizing the tendency of Ca^{2+} to bond two neighboring DPG molecules. The positive charges of cytochrome *c* are more widely dispersed, permitting interaction with two or more DPG molecules. In the inner mitochondrial membrane DPG, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine are all present (Parsons et al., 1967). There is no evidence from the spin-labeling experiments presented

here that cytochrome *c* can cause gross phase separations of these lipids in the inner mitochondrial membrane. These experiments do demonstrate in an interesting way the interaction of cytochrome *c* with the negatively charged lipid, DPG. We speculate that there is a nonrandom distribution of DPG in the inner mitochondrial membrane with DPG-rich regions about the intrinsic electron transport proteins. There is evidence that cytochrome oxidase tightly binds some DPG (Awasthi et al., 1970) and that there is in addition a boundary layer or halo of lipid about cytochrome oxidase (Jost et al., 1973) that may consist partly of DPG. The DPG would then attract the cytochrome *c* through electrostatic interactions and confine it to the region of the cytochrome b–c_1 and cytochrome oxidase complexes, increasing the rate of electron transport.

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