BINDING AND ELECTRON TRANSFER TO CYTOCHROME c IN ARTIFICIAL PHOSPHOLIPID MEMBRANES

Harold K. Kimelberg and Chuan-pu Lee
Johnson Research Foundation, School of Medicine
University of Pennsylvania
Philadelphia, Pa. 19104

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SUMMARY

The binding characteristics of cytochrome c to mixed cardiolipin lecithin membrane systems are described. This binding is reduced by increasing the ionic strength or by omitting cardiolipin. Only some 10 to 20% of the cytochrome c is reduced by ascorbate, and dithionite gives only a small further increase. The remaining cytochrome c is, however, rapidly reduced if TMPD or PMS is added. The relationship of these findings to work on mitochondrial systems is discussed, and an interpretation based on the permeability characteristics of the membrane is proposed.

Cytochrome c is distinguished from the other cytochrome components of the respiratory chain in being readily washed out from swollen mitochondria at high ionic strengths (1,2). Conversely, the cytochrome c present in submitochondrial particles, prepared from mitochondria by sonication, is only extracted after treatment with phospholipase (3), or surface active agents in the case of Keilin-Hartree submitochondrial particles (4). The early work of both Slater (5) and Tsou (6) had sharply distinguished between the reactivity and catalytic activity of the "endogenous" cytochrome c of Keilin-Hartree submitochondrial particles, and soluble, "exogenous" cytochrome c which is able to react with it. This communication reports preliminary experiments on the binding of cytochrome c to phospholipid vesicles (7), in an attempt to determine which features of the phospholipid membrane can account for phenomena described in studies on mitochondria and submitochondrial particles.

MATERIALS AND METHODS

Lecithin and cardiolipin were obtained from either the Sylvana Company, New Jersey, or Supelco, Inc., Bellafonte, Pennsylvania, and used without further purification. Cytochrome c was Type III from Sigma.

Lubrol was kindly supplied by Dr. Ernesto Carafoli, University of Modena, Italy. Phospholipid liquid crystals or vesicles were prepared essentially according to the method of Bangham et al. (7). Mixtures of the phospholipids, after evaporation of solvent, were swollen in 0.015 M potassium chloride in the presence of cytochrome c, with mechanical shaking (pH = 7.8 - 8.5). After centrifugation at 30,000 g for 10 minutes the pellet was then washed twice with 0.15 M potassium chloride with resuspension and re-centrifugation between washes, after which no cytochrome c was spectrophotometrically detectable in the supernatant. The pellet was then taken up in a small volume of 0.15 M potassium chloride by a brief homogenisation. The pH of the pellet was 6.0 - 6.5. The concentration of cytochrome c in the pellet was determined by reduced minus oxidized difference spectra in a split-beam spectrophotometer (E_{mM} $_{550-540}$ = 19). For kinetic measurements of cytochrome c reduction, a dual-wavelength recording spectrophotometer was used. The reaction medium used was 0.15 M potassium chloride, buffered with 0.01 M sodium succinate to a final pH of 7.5.

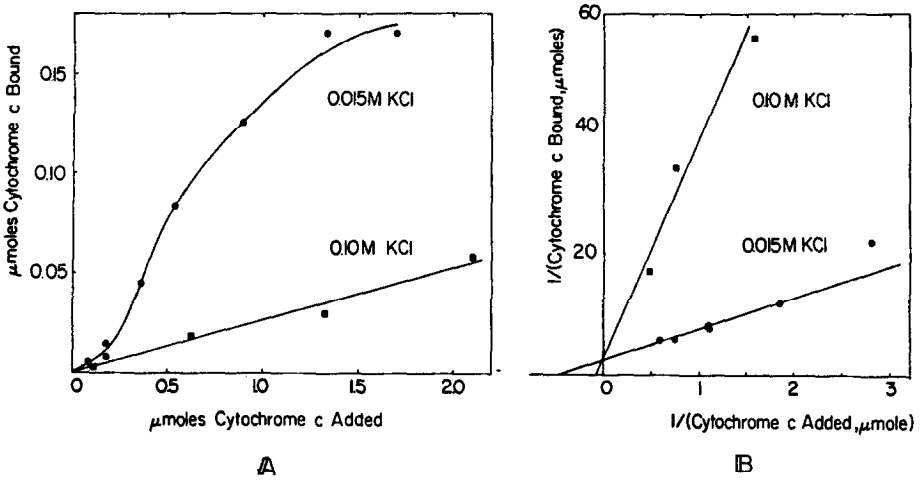

Fig. 1. Binding of Cytochrome c to Cardiolipin-Lecithin Vesicles and the Effect of Ionic Strength.
Vesicles prepared as described under materials and methods. Concentrations of KCl refer to initial swelling medium. Vesicles then washed twice in 0.15 M KCl. Quantities of lipids used were 8 mg lecithin plus 2 mg cardiolipin, finally suspended in 1 ml. 0.15 M KCl. Fig. B is a double reciprocal plot of Fig. A using the values for concentrations of added cytochrome c greater than 0.3 µmoles.

RESULTS

The binding curve of cytochrome c and the phospholipid vesicles, prepared as described, is shown in Figure 1A. The effect of initially swelling the phospholipids and cytochrome c in the presence of 0.10 M potassium chloride, rather than 0.015 M potassium chloride is also included. It is clear that the binding of cytochrome c is decreased at the higher ionic strength in an analogous manner to mitochondria, and suggests that binding in both cases is primarily electrostatic. In support of this a double reciprocal plot of binding at the two potassium chloride concentrations shows that the inhibition is competitive (Figure 1B). Because of the sigmoidal nature of the binding curve at 0.015 M potassium chloride, only the values at higher concentrations of added cytochrome c are used, to give a linear plot. The sigmoidal nature of the curve may be due to an interaction between cytochrome c and the phospholipids, so that the presence of cytochrome c modifies the structure of the vesicles (8).

The ratio of cardiolipin to lecithin used throughout this work was 1:4. This was chosen to approximate the relative concentrations of

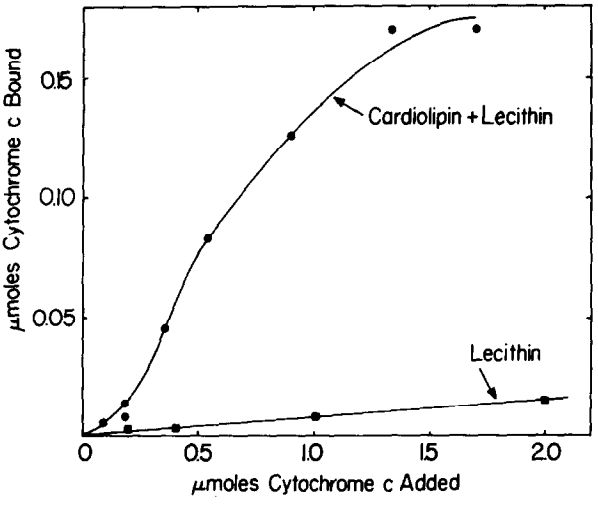

Fig. 2. Comparison of the Binding of Cytochrome c to Mixed Cardiolipin-Lecithin Vesicles and Vesicles Containing Only Lecithin. Conditions as in Fig. 1. Quantities of lipids used were 8 mg lecithin plus 2 mg cardiolipin, or 10 mg lecithin.

Abbreviations: TMPD = N:N:N':N' tetramethyl p-phenylenediamine, PMS = phenazine methosulfate.

cardiolipin to lecithin plus phosphatidylethanolamine, found in the inner membrane of mitochondria (9). The inner membrane of mitochondria is in turn distinguished from other membranes in its unusually high level of cardiolipin. Figure 2 shows that the omission of cardiolipin markedly lowers the binding of cytochrome c. These preparations, containing only lecithin, still show a differential reducibility to ascorbate and TMPD.

Since the cytochrome c present in the final pellet was resistant to further extraction by 0.15 M potassium chloride, it is analogous to the endogenous cytochrome c of sonicated submitochondrial particles (10), or excess, soluble cytochrome c trapped inside sonicated submitochondrial particles (11). Figure 3A shows the reduction of this cytochrome c by ascorbate, as measured in the double-beam spectrophotometer. It can be clearly seen that only a fraction of the cytochrome c is readily

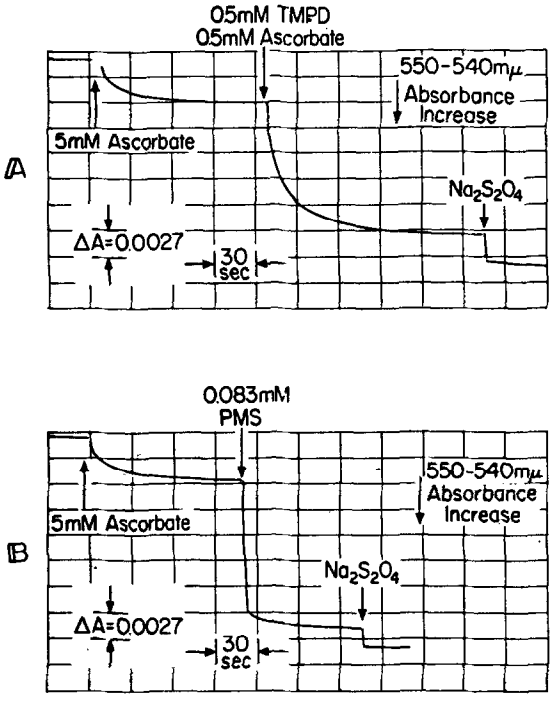

Fig. 3. Reduction of Cytochrome c Bound to Cardiolipin-Lecithin Vesicles by TMPD and PMS Compared to Ascorbate.
A and B: 4.1 mμ moles cytochrome c per mg total lipid, suspended at a final concentration of 0.61 mg total lipids in 3 ml, 0.15 M KCl, 0.01 M succinate, pH 7.5. Similar results were obtained with 50 mM potassium phosphate, 1 mM EDTA, pH 7.3. Ratio of lecithin:cardiolipin was 4:1. Reagents added to the final concentrations shown. Dithionite added (1-3 mg) as solid.

reducible by ascorbate. A much greater fraction, however, is reducible by TMPD. This corresponds essentially to Slater's findings (5) for the endogenous cytochrome c of Keilin-Hartree particles. Figure 3B shows that PMS also reduces the cytochrome c that is not reduced by ascorbate. Figure 4A illustrates that dithionite, in the absence of PMS or TMPD, reduces the ascorbate non-reducible cytochrome c very slowly. By adding lubrol at a final concentration of 0.01%, the amount of cytochrome c reduced by ascorbate was slowly increased (Fig. 4B). No differences in the α, β or γ peaks of the fully reduced minus oxidized difference spectrum of cytochrome c incorporated into phospholipid vesicles, compared to the difference spectrum of soluble cytochrome c, could be detected.

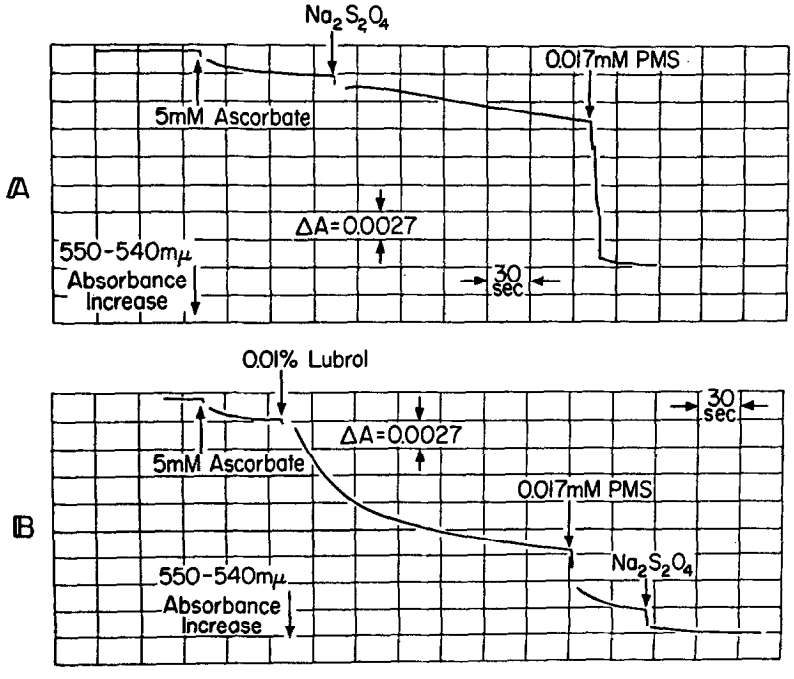

Fig. 4. Reduction of Cytochrome c Bound to Vesicles by Dithionite and the Effect of Lubrol.
A. 14.2 mμ moles cytochrome c per mg total lipids, suspended at a final concentration of 0.26 mg lipids in 3 ml 0.15 M KCl, 0.01 M succinate, pH 7.5. Ratio of lecithin:cardiolipin was 4:1. Dithionite added as solid (1.5 mg).
B. Conditions as in A.

DISCUSSION AND CONCLUSION

There are certain similarities and differences between the work reported in this communication and the studies on cytochrome c - lipid complexes by Das et al. (12), Das and Crane (13), and Reich and Wainio (14). Since the cytochrome c - lipid complexes were in some cases (12,13) extracted into an organic phase, it is not clear whether they would be structurally similar to the completely aqueous, liquid crystal structures employed in the present study. All the above papers described only the binding characteristics of these complexes including the effect of variations in the phospholipid composition (13), and the addition of surface active agents (14) or cations (13). The finding that much less of the acidic phospholipids were bound to cytochrome c relative to other phospholipids (12,13) is supported by the present finding that cardiolipin greatly increases the amount of cytochrome c bound to the sedimented vesicles.

The cytochrome c bound in the final pellet, which is resistant to further extraction by high concentrations of potassium chloride, resembles the non-extractable cytochrome c of sonicated submitochondrial particles (10), and the soluble cytochrome c that can be trapped inside sonic particles (11). It is distinct from the readily extractable cytochrome c of mitochondria (1,2), which, it has been suggested, implies a binding site for cytochrome c on the outside surface of the inner mitochondrial membrane (11,15,17). The majority of the cytochrome c incorporated into phospholipid vesicles, in its inability to be reduced by ascorbate, also resembles the endogenous cytochrome c of Keilin-Hartree particles (5), and soluble cytochrome c trapped inside sonicated submitochondrial particles (16). This indicates similarities between the mitochondrial membrane, and the artificial membranes described here.

At least two interpretations are possible for these differences in reducibility between the submitochondrial particle or artificial membrane-bound cytochrome c, and soluble cytochrome c. One is that a change in conformation of cytochrome c is induced by its binding to membranes, resulting in a change in its chemical reactivity. The alternative is that cytochrome c is located inside a closed vesicular structure, and is therefore sequestered behind a membrane permeability barrier. At present the latter interpretation is favoured in view of the slow reducibility by dithionite. Furthermore, phospholipid vesicles have a very low perme-

ability to large anions such as phosphate (7,18) and by analogy to the $S_2O_4^{=}$ or ascorbate anion. The inability of cytochrome c to diffuse through or penetrate the vesicle membranes is indicated by bound cytochrome c having no effect on K^+, Na^+ or Cl^- diffusion in these systems (19).

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