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Interactions of Cytochrome *c* with Phospholipid Membranes

II. Reactivity of Cytochrome *c* Bound to Phospholipid Liquid Crystals

H. K. KIMELBERG* and C. P. LEE

Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Summary. Cytochrome *c* can be bound to mixed cardiolipin-lecithin liquid crystals so that it cannot be removed by repeated washings with solutions of high ionic strength. The oxidized and reduced spectra of this cytochrome *c* show no detectable differences from those for soluble cytochrome *c*. Unlike soluble cytochrome *c*, however, some 90% of the bound cytochrome *c* is not reduced by ascorbate, and it is only slowly reduced by dithionite. The addition of redox dyes causes complete and immediate reduction in the presence of ascorbate or dithionite. It is suggested that this is because the dyes possess some degree of lipid solubility and are able to penetrate the phospholipid membrane barriers separating cytochrome *c* from the bulk solution. The addition of detergents, such as Triton X-100, also promotes reduction of the bound cytochrome *c* by ascorbate. A small change in the standard potential from 273 mV for soluble cytochrome *c* to 225 mV for the bound cytochrome *c* was found. The bound cytochrome *c* reacts readily with potassium cyanide to form the normal cyanide-ferricytochrome *c* complex, differing in the rate of formation from soluble cytochrome *c* only at alkaline pH values. The relationship of these findings to work on the membrane-bound cytochrome *c* in mitochondria and submitochondrial particles is discussed.

In paper I of this study (Kimelberg, Lee, Claude & Mrena, 1970), we described a method of binding cytochrome *c* to phospholipid liquid crystals (Bangham, Standish & Watkins, 1965) at low ionic strength, such that a significant portion of the cytochrome *c* enclosed inside the liquid crystalline membranes could not be removed by repeated washings at high ionic strength. In this respect, cytochrome *c* in these model membrane systems resembles the endogenous cytochrome *c* of Keilin-Hartree submitochondrial particles (Tsou, 1952), the endogenous cytochrome *c* of submitochondrial particles prepared by sonication (Lenaz & MacLennan, 1966) and soluble cytochrome *c* trapped inside sonicated submitochondrial particles (Lee & Carlson, 1968;

* Present address: Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14203.

Lee, *in press*). The endogenous cytochrome *c* of mitochondria, on the other hand, after a brief exposure to hypotonic conditions, is readily extracted by washing at high ionic strength (Schneider, Claude & Hogeboom, 1948; Jacobs & Sanadi, 1960).

The endogenous cytochrome *c* of Keilin-Hartree particles has classically been distinguished from soluble "exogenous" cytochrome *c* with regard to its reactivity and catalytic activity. Slater (1949) found that the half-time for the reduction of the endogenous cytochrome *c* of Keilin-Hartree particles by ascorbate under anaerobic conditions was much longer than for soluble cytochrome *c*, and Tsou (1952) reported that this endogenous cytochrome *c* did not form a ferricytochrome *c*-cyanide complex.

This paper reports experiments investigating the reactivity of cytochrome *c* bound in phospholipid liquid crystals toward ascorbate and cyanide, as well as conditions modifying the response. Redox titrations of the bound cytochrome *c* are also included.

Materials and Methods

The method of preparation of phospholipid liquid crystals containing cytochrome *c* is described in paper I (Kimelberg *et al.*, 1970). Redox titrations were performed under nitrogen in an anaerobic cuvette designed by Dr. P.L. Dutton of this department. Potentials were directly measured with a platinum-calomel electrode couple with a suitable pH meter, and the reduction of cytochrome *c* was simultaneously monitored with a double-beam recording spectrophotometer, as described by Caswell and Pressman (1968). A magnetic stirrer arrangement provided continuous mixing. Wurster's blue was kindly supplied by Dr. Tsoo E. King of the State University of New York at Albany. All other chemicals were of the purest quality available commercially.

Results

Fig. 1 shows an oxidized and reduced absolute spectrum of the cytochrome *c* bound to phospholipid liquid crystals or vesicles. The reference cuvette contains phospholipid without cytochrome *c* to offset light scattering from the turbid suspension. As can be seen, a slow drift is still obtained toward shorter wavelengths, although the two solutions were matched in absorbance at 600 nm. This may imply that the light-scattering properties of the two solutions were not identical, which is suggested by the structural changes caused by binding cytochrome *c* to the phospholipid vesicles, described in paper I (Kimelberg *et al.*, 1970). Nonetheless, it is apparent that no change can be detected in the positions or relative magnitude of the spectra when compared to soluble cytochrome *c* (Margoliash & Frohwirt,

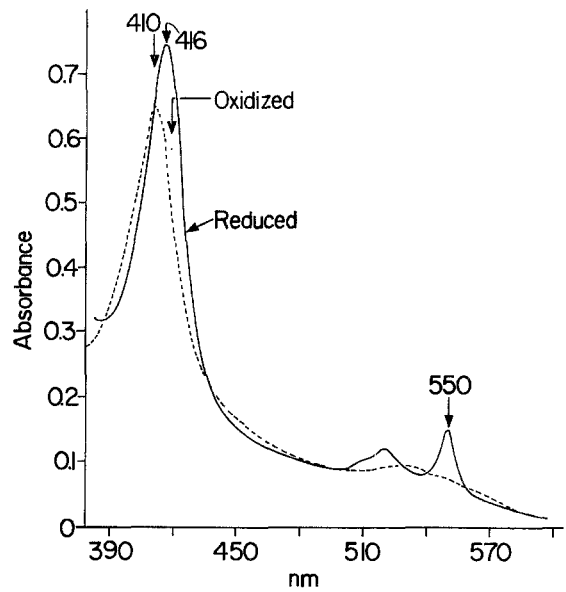

Fig. 1. Oxidized and reduced absolute spectra of cytochrome *c* bound in phospholipid vesicles. Cytochrome *c* in phospholipid vesicles at a concentration of 12.6 nmoles cytochrome *c*/1.16 mg lecithin *plus* 0.34 mg cardiolipin was added to 0.05 M potassium phosphate (pH 7.3) in the sample cuvette. An equal quantity of phospholipids without cytochrome *c* was added to the reference cuvette. The sample was reduced with final concentrations of 4.7 mM ascorbate, 7.8 μM DCPIP, and 2–3 mg dithionite. Final volume: 3.2 ml. Temperature: 25 °C

1959; Butt & Keilin, 1962). A similar lack of any change has also been noted by others (Gulik-Krzywicki, Shechter, Luzzati & Faure, 1969). The absorbance maxima are at 550, 520 and 416 nm in the reduced form, and at 527 and 410 nm in the oxidized form. Isosbetic points from Fig. 1 are at 558, 541, 529, 508, 438 and 410 nm. The ratio of reduced/oxidized absorbance for $\frac{\Delta A \text{ red.}\, 416-438}{\Delta A \text{ oxid.}\, 410-438}$ was 1.23. The absorbance difference was used for this ratio because of the light-scattering increment at smaller wavelengths.

The reduction of cytochrome *c* by ascorbate, when bound in phospholipid vesicles, is shown in Fig. 2. The reduction is recorded using a double-beam recording spectrophotometer set at 550 minus 540 nm. It can be seen that less than 10% of the total cytochrome *c* is reduced by ascorbate alone. The addition of phenazine methosulfate (PMS), however, as shown in Fig. 2A, causes immediate and complete reduction. This may be because PMS acts as a lipid-soluble "shuttle" for electrons from ascorbate in the bulk solution to cytochrome *c* enclosed inside the phospholipid membrane barriers, assuming, as might be expected, that the membranes are impermeable to

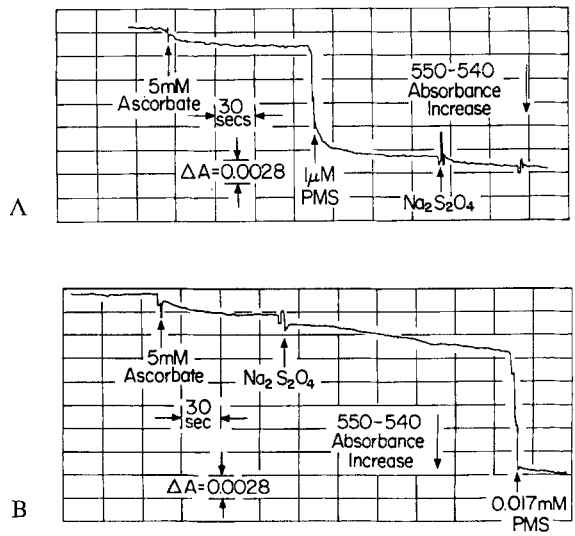

Fig. 2A and B. Reduction of cytochrome *c* bound to phospholipid vesicles by ascorbate and dithionite and the effect of PMS. A. 10.7 nmoles cytochrome *c*/mg total lipids, suspended at a final concentration of 2.67 nmoles cytochrome *c*/0.25 mg phospholipids, was added to 3 ml 0.15 M KCl, 0.01 M sodium succinate, pH 7.5, 25 °C. Ratio of lecithin/cardiolipin was 4:1 by weight. Final concentrations of reagents were as shown; dithionite was added as solid (2 – 3 mg) to give a final concentration of 3.9 – 5.1 mM. B. 14.2 nmoles cytochrome *c*/mg total lipids, suspended at a final concentration of 0.26 mg lipids in 3 ml 0.15 M KCl – 0.01 M sodium succinate, pH 7.5, 25 °C. Ratio of lecithin/cardiolipin was 4:1 by weight. Dithionite was added as solid (1.5 mg) to give a final concentration of 2.9 mM

ascorbate. In this experiment, 1 μM PMS was used, but additions of PMS as low as 0.05 μM at these lipid concentrations can be used before a decrease in the rate and extent of cytochrome *c* reduction is observed. Fig. 2B shows that dithionite (sodium hydrosulfite), in the absence of a lipid-soluble electron mediator such as PMS, will reduce cytochrome *c* only very slowly. The final concentration of dithionite used in this experiment was 2.9 mM. Increasing the concentration of dithionite fourfold increases the rate of reduction by a factor of six, whereas increasing the ascorbate concentration up to 20 mM had no effect on the reduction of cytochrome *c*. Other redox dyes also promoted cytochrome *c* reduction but at somewhat higher concentrations. Both dichlorophenol indophenol (DCPIP) and tetramethyl *p*-phenylenediamine (TMPD) were found to be effective.

Reduction of cytochrome *c* by ascorbate could also be promoted by the addition of detergents. Fig. 3 shows a titration of this response by Triton X-100. Complete reduction of cytochrome *c* is obtained with an amount of

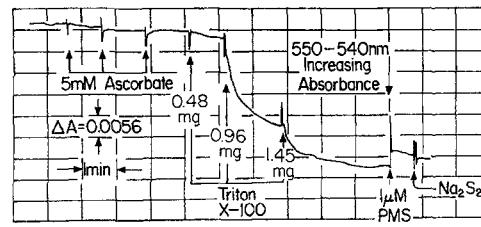

Fig. 3. Effect of Triton X-100 on reduction of cytochrome *c* in phospholipid vesicles by ascorbate. 5.85 nmoles cytochrome *c*/1 mg lecithin *plus* 0.13 mg cardiolipin was added to 3 ml 0.15 M KCl – 0.01 M succinate, pH 7.5, 28 °C. Triton X-100 was added in successive amounts of 0.48 mg; the amounts shown in the figure refer to the final concentrations after these additions

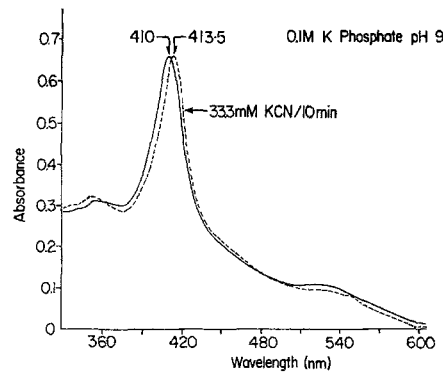

Fig. 4. Absolute spectrum of cyanide complex with ferricytochrome *c* bound in phospholipid vesicles. 14.6 nmoles cytochrome *c* plus 1.9 mg total phospholipid in sample cuvette and 2.1 mg phospholipid in reference cuvette in 3 ml 0.1 M potassium phosphate, pH 9.0, 25 °C. 33.3 mM potassium cyanide, final concentration, left for 10 min before scan was commenced. Ratio of cardiolipin/lecithin was 1:4.5 by weight

Triton X-100 (1.45 mg) approximately equivalent to the amount of phospholipids (1.13 mg), on a weight basis.

As mentioned in the introduction to this paper, Tsou (1952) had qualitatively distinguished between isolated soluble cytochrome *c* and the endogenous cytochrome *c* of a particulate preparation derived from mitochondria on the basis that the endogenous ferricytochrome *c* did not form a complex with cyanide. Fig. 4 shows the absolute spectrum, obtained as described for Fig. 1, of ferricytochrome *c* bound in phospholipid vesicles in the presence and absence of 33.3 mM KCN at pH 9.0. Although the shift in the α-peak region (Potter, 1941) at 540 to 530 nm cannot be readily discerned because of interference from light scattering, a shift of the Soret peak from 410 to 413.5 nm, characteristic of the cyanide complex (Tsou, 1952), can be observed.

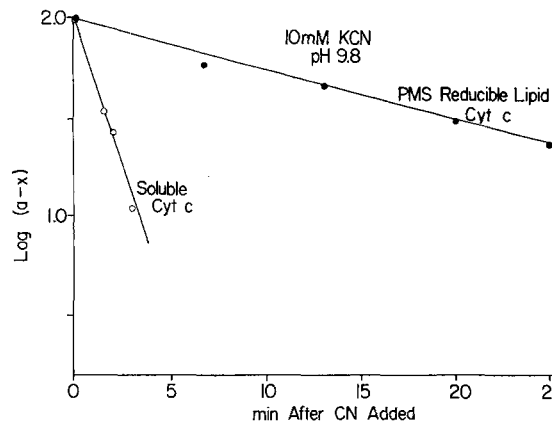

Fig. 5. First-order plots for the rates of reaction of cyanide with soluble ferricytochrome *c* bound in phospholipid vesicles. 14.2 nmoles cytochrome *c*/mg total lipids, suspended at a final concentration of 0.410 mg lecithin *plus* 0.115 mg cardiolipin in 3 ml 0.15 M KCl – 0.02 M Tris-chloride, pH 9.8, 25 °C, to give a final concentration of 2.5 μM cytochrome *c*. The pH of potassium cyanide was adjusted to the pH of the experiment. Soluble cytochrome *c* was at a final concentration of 2.6 μM. Cytochrome *c* in both cases was reduced with 5 mM ascorbate *plus* 1 μM PMS at various time intervals after addition of cyanide. *a* represents the percent of cytochrome *c* reduced in the absence of cyanide, and *x* the percent that cannot be reduced after the addition of cyanide, which corresponds to the amount of cytochrome *c* in the cyanide complex

Since cytochrome *c* inside the phospholipid vesicles apparently does react with cyanide, it was of interest to compare the kinetics of this reaction with that of soluble cytochrome *c*. It was found that PMS was unable to reduce soluble cytochrome *c* in the cyanide-cytochrome *c* complex. This provided a means of comparing the kinetics of the cyanide-cytochrome *c* reaction in both cases. As can be seen in Fig. 5, the reaction shows first-order kinetics for both soluble and lipid-bound cytochrome *c*, and, at this pH (9.8), a clear difference in rates is obtained. The first-order constant for soluble cytochrome *c* was 6.8×10^{-1} min^{-1}, and for cytochrome *c* bound to phospholipid it was 5.8×10^{-2} min^{-1}. The second-order constants derived from these figures and other similar plots, and the effect of varying the pH is shown in the Table.

The absorption spectrum of cytochrome *c* is relatively insensitive to subtle changes in the state of the molecule. Since changes in redox behavior might be a more sensitive indicator of any alterations, the standard redox potentials of cytochrome *c* bound in phospholipid vesicles were measured to determine if any difference existed between the free and bound protein. The potentials were measured in a closed cuvette under a continuous pressure of nitrogen gas. Ascorbate was titrated in as a source of reducing

Table. *Rates of the reaction of soluble cytochrome c and phospholipid-bound cytochrome c with* 10 mM *cyanide*[a]

pH	Rate constant (M^{-1} min^{-1})	
	Soluble *c*	Phospholipid-bound *c*
6.35	2.6	1.8
7.45	5.8	3.2
8.60	57.5	17.7
9.80	67.7	5.8

[a] 0.15 M KCl *plus* 0.01 M sodium succinate buffer used at pH 6.35 and 7.45; 0.02 M Tris-chloride, instead of 0.01 M succinate, used at pH 8.6 and 9.8. Final concentrations of cytochrome *c* used were in the range 1.6 – 2.5 μM. 25 °C.

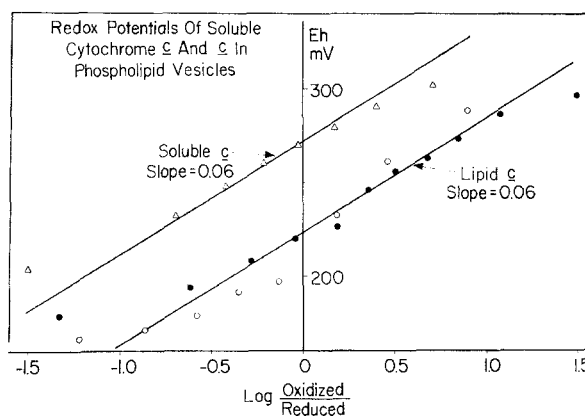

Fig. 6. Redox potentials of soluble cytochrome *c* and cytochrome *c* in phospholipid vesicles. 14.6 nmoles cytochrome *c*/1.9 mg phospholipid was added to 6 ml 0.15 M KCl – 0.01 M sodium succinate, pH 7.5, 22 °C, to give a final concentration of 2.0 μM cytochrome *c*. Soluble cytochrome *c* was added to a final concentration of 1.3 μM. Samples of 0.83 μM sodium ascorbate were added for the experimental points given by ▵ and •. Samples of 1.67 μM sodium ascorbate were added for the points shown as ○. Wurster's blue was present at a concentration of 8.3 μM. Reduction was measured at 416 – 434 nm. The potential was measured directly, utilizing a platinum-calomel electrode couple with a Radiometer pH meter type 22. The readings were made in a closed cuvette under a constant pressure of nitrogen. Mixing was done by a magnetic stirring bar positioned to one side of the cuvette

equivalents. Wurster's blue was present as a mediator between cytochrome *c* and the platinum electrode (Caswell & Pressman, 1968), as well as between ascorbate and phospholipid-bound cytochrome *c* (Kimelberg & Lee, 1969). Both the potential and the reduction state of cytochrome *c* were simultaneously measured, as described in Methods. Fig. 6 shows a plot of the logarithm

of the oxidized-to-reduced ratio vs. the potential in millivolts. Using the normal equation for the relationship between the measured electrode potential (E_h) and the standard potential (E_0'):

$$E_h = E_0' + 2 \cdot 303 \frac{RT}{nF} \log \frac{[\text{Ox}]}{[\text{Red}]}.$$

The intercept on the ordinate is equal to the standard potential which was found from Fig. 6 to be 273 mV for soluble cytochrome *c* and 225 mV for phospholipid-bound cytochrome. Experimental points from two different experiments are shown for phospholipid-bound cytochrome *c*. In both curves, the slope of the line, equal to $2 \cdot 303\ RT/nF$, was 0.06 giving a one-electron transfer reaction where $n = 1$.

Discussion

Cytochrome *c* incorporated into phospholipid liquid crystals, in its inability to be reduced by ascorbate, resembles the endogenous cytochrome *c* of Keilin-Hartree submitochondrial particles (Slater, 1949) and the soluble cytochrome *c* trapped inside sonicated submitochondrial particles (Lee & Carlson, 1968; Lee, Kimelberg & Johansson, 1969; Lee, *in press*). This indicates similarities between the effects of binding to mitochondrial membranes and the artificial phospholipid membranes described here. A surprising finding, however, is that dithionite reduces cytochrome *c* bound in the phospholipid membranes very slowly, distinguishing it from cytochrome *c* bound to mitochondrial membranes. This suggests that the main barrier to ascorbate and dithionite reduction is the permeability of the phospholipid membranes, since cytochrome *c* normally is immediately reduced by dithionite. These membranes have a low permeability to relatively large anions such as phosphate (Bangham *et al.*, 1965; McGivan & Chappell, 1968), and by analogy to the $S_2O_4^{--}$ and ascorbate anions. The inclusion of acidic phospholipids further lowers anion permeability, sometimes to the very slow rates found for cations (Papahadjopoulos & Watkins, 1967).

It is possible, of course, that the binding of cytochrome *c* to a lipid membrane induces a conformational change in the protein, altering its reactivity towards these reducing agents. A small change in the redox mid-potential of 48 mV more negative for phospholipid-bound cytochrome *c* than for soluble cytochrome *c* was detected, which may indicate such an alteration. A change of this magnitude, however, would have little or no effect on reduction by ascorbate and dithionite since their standard redox potentials are 0.058 and -1.13 mV, respectively. Thus, a membrane per-

meability barrier as discussed above would appear to be the most suitable explanation of the modifications in cytochrome *c* reactivity when bound to phospholipid model membranes, and by analogy when bound to natural membranes of mitochondrial origin. The inability of cytochrome *c* to diffuse through or penetrate the phospholipid membranes is indicated by cytochrome *c* having no effect on K^+, Na^+ or Cl^- diffusion in phospholipid liquid crystals (Papahadjopoulos & Watkins, 1967). It would appear, however, that the natural membranes are far more "leaky" than the model membranes, perhaps owing to the high proportion of protein in the former. This may explain the difference in rates of reduction by dithionite. The alternative is that dithionite, in making a solution anaerobic, allows an endogenous source of reducing equivalents to reduce cytochrome *c* in submitochondrial particles.

The titration of the ascorbate response of cytochrome *c* bound to phospholipid vesicles by Triton X-100 (*see* Fig. 3) is consistent both with a lysing of the membrane structure or a dissociation of the lipid-cytochrome *c* complex (Reich & Wainio, 1961), and consequent reversal of the changes induced by binding. It does emphasize, however, that the failure of cytochrome *c* to be reduced by ascorbate depends on the integrity of the membrane structure.

These observations support the conclusions put forward in paper I that cytochrome *c* was adsorbed onto the surface of the individual lamellae, and probably not on the outside of the outermost layer. These conclusions were based on the failure to extract from the phospholipid vesicles at high ionic strength about 50% of the cytochrome *c* initially bound, and the effect of cytochrome *c* in increasing the width of each membrane.

Because cytochrome *c* bound to phospholipid membranes and the endogenous cytochrome *c* of Keilin-Hartree submitochondrial particles resemble each other in failing to react with ascorbate, it was of interest to examine the reaction of phospholipid-bound cytochrome *c* with cyanide, since Tsou (1952) had stated that endogenous cytochrome *c* failed to react with cyanide. In this regard, a clear distinction between the two types of membrane-bound cytochrome *c* is seen; cytochrome *c* bound in phospholipid membranes does react. No reconciliation of this discrepancy is yet possible, but it does appear that soluble cytochrome *c* bound inside sonicated submitochondrial particles, as well as the endogenous cytochrome *c* in these particles, also react with cyanide (Hsia & Lee, *unpublished observations*). At neutral and acid pH values, very little distinction in the rates of the reaction is seen between soluble cytochrome *c* and cytochrome *c* in phospholipid membranes. At more alkaline pH values, however, the two rates begin to

differ (*see* Table). Since CN^- is the dominant reactive species by several orders of magnitude (George & Tsou, 1950), it may be that the membrane permeability barrier becomes rate limiting only when CN^- is present in kinetically significant quantities. Alternatively, cytochrome *c* in the phospholipid vesicles may be in a milieu of pH 6 to 7.

In conclusion, it has been demonstrated that the properties of soluble cytochrome *c* are altered when it is bound in artificial phospholipid membrane systems. These alterations are consistent with the effects resulting from cytochrome *c* being sequestered behind closed membrane permeability barriers, although small changes in the molecule resulting from binding to the membranes are also indicated.

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