# ASSOCIATION OF CARDIOLIPIN AND CYTOCHROME OXIDASE

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SUMMARY

A small amount of cardiolipin (diphosphatidyl glycerol) is tightly bound to cytochrome oxidase. The stoichiometry approaches one molecule of cardiolipin per molecule of cytochrome a. Solvent extraction and phospholipase treatments which remove or destroy other phospholipids and the bulk cardiolipin do not effect this tightly bound cardiolipin. Potential oxidase activity and membrane formation potential are lost when the tightly bound cardiolipin is removed. This cardiolipin is very similar in fatty acid composition to the total mitochondrial cardiolipin.

Selective extraction studies reveal a specific association of cytochrome oxidase and cardiolipin regardless of the method used for preparation of the oxidase. This lipid is more tightly bound than the bulk of the phospholipid usually found in cytochrome oxidase preparations and approaches a stoichiometric 1:1 association with the oxidase. The cardiolipin oxidase complex has very low activity, but maximum cytochrome oxidase activity can be restored by addition of mitochondrial phospholipids or certain detergents. After partial extraction of the tightly bound cardiolipin, maximal activity cannot be restored by detergents.

Fleischer et al. (1) have studied the phospholipid composition of cytochrome oxidase from beef heart mitochondria and found it to be richer in cardiolipin than other submitochondrial particles. In contrast, Horie et al. (2) and Morrison et al. (3) found a purified cytochrome oxidase from pig heart mitochondria, prepared by repeated fractionation

with cholate-ammonium sulphate, to be practically devoid of phospholipids. Another 'lipid free' preparation made with the non-ionic detergent Triton X-100 and X-114 has been reported (4,5). The present communication deals with the presence and characteristics of tightly bound cardiolipin in the latter preparation.

## Methods

Beef heart mitochondria (BHM) were prepared by method of Löw and Vallin (6). 'Lipid free' cytochrome oxidase was prepared and assayed according to procedures reported earlier (4,5). Phosphorous was estimated according to Chen et al. (7) and protein determinations were made by the methods of Yonitani (8) and Lowry (9).

For studies of fatty acid composition and distribution, mitochondrial and cytochrome oxidase preparations were extracted twice with chloroform: methanol, (2:1, v/v), once with ethyl ether:ethanol (1:1, v/v) and finally twice with chloroform:methanol:aqueous ammonia (7:1:5%). Methods for separation and determination of the distribution and fatty acid composition of phospholipids were identical to those used previously (10) except that in the present study, dichlorofluorescein was used to visualize lipids for subsequent fatty acid analysis.

## RESULTS AND DISCUSSIONS

The distribution of phospholipids (based on percentage of total lipid phosphorus) of beef heart mitochondria (BHM) and various cytochrome oxidase preparations is given in Table I. In addition to constituents listed, traces of lysophosphatidyl ethanolamine were also observed in mitochondria. Whereas the lipid composition of mitochondria described here is similar to that reported by Fleischer et al. (16), the total percentage of lipid phosphorus accounted for by the three major phospholipids (PE, PC, DPG) in this study was 85 percent as compared with a value of 96 percent reported by Fleischer and Rouser (16). The presence

Table I

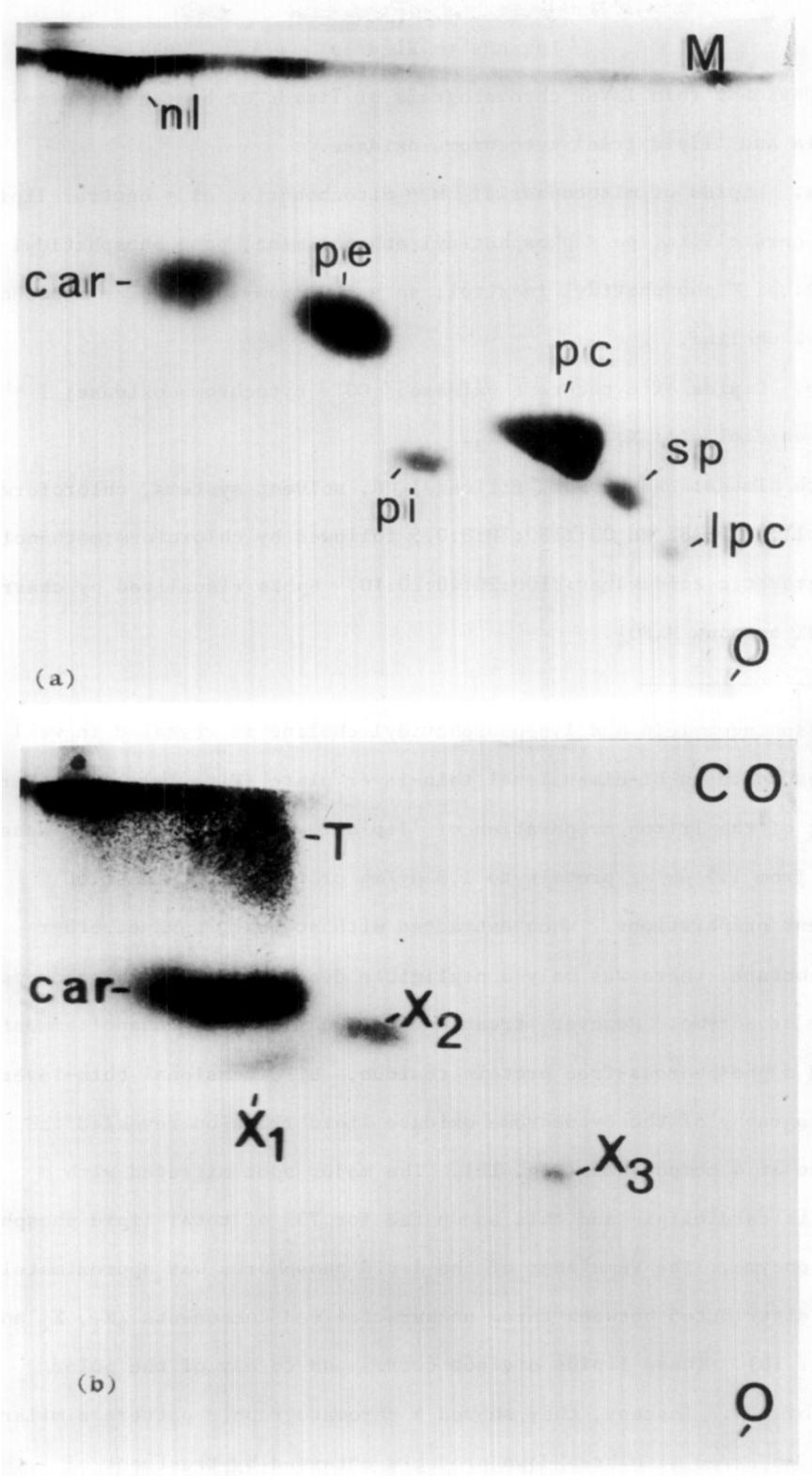
Phospholipid Distribution in Beef Heart Mitochondria and Cytochrome Oxidase Preparations

| Phospholipid | Mitochondria (19.5 μg P/mg protein) | cytochrome oxidase | | |
|---|---|---|---|---|
| | | Triton prep. (1.59 μg P/mg protein) | Cholate-deoxycholate prep.* (15.7 μg P/mg protein) | Reconstituted from Triton* prep. with phospholipid (20.0 μg P/mg protein) |
| PE | 29.8 | 0 | 30.6 | 30.0 |
| PC | 37.8 | 0 | 32.0 | 34.0 |
| DPG | 18.2 | 73.0 | 30.0 | 30.0 |
| PI | 5.4 | 0 | ---- | ---- |
| LPC | 2.8 | 0 | ---- | ---- |
| Others | 6.0 | 27 | 8 | 6 |

*Prepared by method of Fowler et al. (11) and dialyzed to remove cholate (12).

*Prepared by sonicating the Triton prep. with an excess of total mitochondrial phospholipid micelles and centrifuging down the membraneous pellet.

PE ≡ Phosphatidyl ethanolamine; PC ≡ phosphatidyl choline; DPG ≡ cardiolipin (diphosphatidyl glycerol); LPC ≡ lysophosphatidyl choline; PI ≡ phosphatidyl inositol.


M
nl
car-
pe
pc
sp
pi
lpc
O
(a)
CO
-T
car-
X2
X1
X3
O
(b)

Legends to Figures

Bi-dimensional thin layer chromatograms of lipids of beef heart mitochondria and 'lipid free' cytochrome oxidase.

Fig. Ia. Lipids of mitochondria: M = mitochondria; nl = neutral lipids; car. = cardiolipin; pe = phosphatidyl ethanolamine; pc = phosphatidyl choline; pi = phosphatidyl inositol; sp = sphingomylein; lpc = lysophosphatidyl choline.

Fig. Ib. Lipids of cytochrome oxidase: CO = cytochrome oxidase; T = Triton; Car. = cardiolipin; $X_1$, $X_2$ and $X_3$.

For both plates: adsorbant ≺ silicagel HR; solvent systems, chloroform: methanol:water:28% $NH_4OH$::130:70:8:0.5 followed by chloroform:methanol: acetone:acetic acid:water::100:20:40:20:10. Spots visualized by charring with 50% aqueous $H_2SO_4$.

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of PI, sphingomylein and lysophosphatidyl choline is revealed in well resolved spots on bi-dimensional thin-layer plate (Fig. Ia). Phosphorous content of the Triton preparation of 'lipid free' cytochrome-c oxidase ranged from 1.5 μg/mg protein to 1.8 μg/mg protein in at least 50 different preparations. When extracted with acetone, hexane, ether or iso-octane, there was only a negligible decrease in the phosphorous content of enzyme. However, treatment with chloroform:methanol:ammonia yielded a phosphorous-free protein residue. Bi-dimensional thin-layer chromatography of the cytochrome oxidase lipid fraction revealed the presence of 4 components (Fig. Ib). The major spot migrated with authentic cardiolipin and this accounted for 73% of total lipid phosphorus of the enzyme. The remainder of the lipid phosphorus was approximately evenly distributed between three uncharacterized components ($X_1$, $X_2$ and $X_3$; Fig. Ib). These lipids did not correspond to any of the polar lipids of BHM. Instead, they showed a chromatographic pattern similar to that observed with breakdown products obtained by treatment of

cardiolipin with N. naja venom phospholipase-A.

When the 'lipid free' cytochrome c oxidase was sonicated with micelles of total mitochondrial phospholipids and centrifuged (40,000 rpm, 40 rotor, Beckman Spinco L-65) a membraneous pellet was obtained which contained 15-20 μg phosphorous/mg protein. This phosphorous level could not be decreased by repeated aqueous washings. However, most of

Table II

Phosphorous Content of 'lipid-depleted' Cytochrome Oxidase and its Extractibility with Different Solvents

| Extracting Solvents | P. μg/mg Protein | |
|---|---|---|
| | Triton prep. | Cholate-deoxycholate prep. |
| None | 1.60 | 20.2 |
| Diethyl ether | 1.65 | 9.4 |
| Hexane | 1.60 | ---- |
| Isooctane | 1.68 | 16.0 |
| Acetone | 1.55 | 3.0 |
| Chloroform:methanol (2:1) | .80 | 2.0 |
| Chloroform:methanol:$NH_4OH$(28%)::100:50:2 | none | none |

the phospholipid could be extracted from this preparation with acetone, leaving a residue having a phosphorous level similar to that of the original lipid deficient enzyme. Phospholipid composition of this 'reconstituted' membraneous cytochrome oxidase was studied along with another membraneous preparation of cytochrome oxidase prepared by method of Fowler, Richardson and Hatefi (11) and freed of cholate-deoxycholate by method of McConnel et al. (12). Results (Table III) indicate that both of these preparations are rich in cardiolipin (DPG) and the results are similar to those reported by Fleischer et al.(1). This is strongly

Table III

Restoration of Activity of Lipid Depleted Cytochrome Oxidase Preparation on Addition of Phospholipids and Detergents

| Treatment | μg P/mg protein | Activity (μmol $O_2$/min/mg) |
|---|---|---|
| Preparation I | | |
| None | 1.59 | 3.39 |
| + BHM phospholipids | ---- | 55.4 |
| + Emasol | ---- | 57.5 |
| + Tween 80 | ---- | 54.8 |
| + Deoxycholate | ---- | 11.8 |
| + Cardiolipin | ---- | 58.4 |
| + Lecithin | ---- | 28.7 |
| + Phosphatidyl ethanolamine | ---- | 19.0 |
| Preparation II | | |
| None | 1.1 | 0.66 |
| + BHM phospholipids | ---- | 32.2 |
| + Emasol | ---- | 20.7 |
| Earlier study (2) | | |
| Tween 80 | 0.2-0.36 | 8.25 |

Preparation I - regular triton preparation

Preparation II - regular triton prep. subjected to repeated fractionations (seven) by sodium cholate (13%) in Tris HCl·1M (pH 7.8) and ammonium sulphate (20% saturation), cf. Horie *et al.* (2).

suggestive of preference for cardiolipin over other mitochondrial lipids. It has been observed (13,14) that, by acetone extraction of BHM, more than 80 percent of the mitochondrial phospholipid can be extracted and that the remaining phospholipid, consisting mainly of cardiolipin can only be extracted on addition of $NH_4OH$ or by extraction with chloroform:methanol:ammonia. Since the amount of cardiolipin required to form a stable complex with cytochrome oxidase cannot account for all the tightly bound cardiolipin of BHM it is suggested that other

stable proteolipid complexes of cardiolipin are also present in mitochondria. Recently Eichberg (15) has isolated a proteolipid from beef heart in which cardiolipin is the major lipid entity.

The phospholipid level (1.5 μg P/mg protein) in this preparation of cytochrome oxidase would be equivalent to 3.5 percent phospholipid and since approximately 73 percent of this is cardiolipin, the cardiolipin content of the enzyme would be about 2.4%. Taking molecular weight of cytochrome oxidase to be 72,000 (16) the molar ratio of cytochrome oxidase to cardiolipin would be 1:1·3 which is close to 1:1. However, if we assume that the uncharacterized constituents $X_1$, $X_2$ and $X_3$ arise from the tightly bound cardiolipin, the molar ratio of bound cardiolipin to the enzyme would approximate 2:1.

This enzyme-lipid complex has only a low activity, but it can be reactivated by incorporation of phospholipids or detergents (Table III). When this 'lipid free' Triton preparation is subjected to repeated fractionation by $NH_4(SO_4)_2$ in Sodium Cholate-Tris HCl buffer (cf. Horie and Morrison[2]) the phosphorous level of cytochrome oxidase attains a minimum value of 1.1 μg P/mg protein. This enzyme, devoid of a part of tightly bound cardiolipin, could be restored to only a fraction of maximal activity by addition of detergents like Emasol. However, on addition of total mitochondrial phospholipids activity is restored to much higher extent (Table III). Thus the lower activity of highly purified preparations of cytochrome oxidase reported by Horie and Morrison (2) and Morrison et al. (3) could be attributed to the removal of tightly bound cardiolipin by repeated fractionation with $(NH_4)_2SO_4$ in the presence of sodium cholate.

Treatment of the lipid deficient oxidase with phospholipase A from Naja naja venom under conditions which cause hydrolysis of 90% of mitochondrial cardiolipid (17) did not cause significant hydrolysis of the tightly bound cardiolipin.

Table IV

Fatty Acid Composition of Mitochondria and

Cytochrome Oxidase Phospholipids (% by weight)

| Acid | Mitochondria | | | | Cytochrome oxidase | |
|---|---|---|---|---|---|---|
| | PC | PE | PI | DPG | DPG | $X_1 + X_2$ |
| 16:0 | 28.5 | 4.9 | 7.0 | 1.7 | 2.9 | 30.7 |
| 16:1 | -- | -- | -- | 2.3 | 2.6 | -- |
| 17:0 | -- | -- | 4.2 | -- | -- | -- |
| 18:0 | 5.2 | 37.6 | 55.6 | 1.1 | 2.3 | 25.1 |
| 18:1 | 23.4 | 5.0 | 8.3 | 7.2 | 7.0 | 28.8 |
| 18:2 | 30.6 | 15.4 | 5.1 | 83.9 | 80.6 | 10.1 |
| 18:3 | 2.7 | 1.1 | 0.6 | 2.3 | 2.4 | -- |
| 20:3 | -- | -- | -- | 0.9 | 0.7 | -- |
| 20:4 | 4.9 | 28.2 | 14.1 | 0.7 | 1.5 | 3.1 |
| 24:0 | 0.9 | 4.1 | -- | -- | -- | -- |
| $T_r$3.05* | 3.3 | 2.2 | 5.1 | -- | -- | -- |
| $T_r$8.18* | -- | 1.6 | -- | -- | -- | -- |
| $T_r$2.29* | -- | -- | -- | -- | -- | 2.2 |

PC - Phosphatidyl choline, PE - Phosphatidyl ethanolamine, PI - Phosphatidyl Inositol, DPG - Diphosphatidyl glycerol (cardiolipin).

*Uncharacterized acids reported as retention time relative to methyl stearate.

In order to determine possible specifity in the fatty acid composition of the tightly bound phospholipids, the fatty acid composition of these lipids was determined along with those of intact mitochondrial phospholipids. Fatty acids analyses (Table IV) indicate, that apart from a slightly higher level of saturated fatty acids in cytochrome oxidase cardiolipin, there is no major difference in the fatty acid patterns of the bound and parent mitochondrial cardiolipin. However, in comparison

with cardiolipin, the minor uncharacterized constituents, $X_1$ and $X_2$, have a totally different fatty acid composition. If $X_1$ and $X_2$ are degradation products of cardiolipin, their fatty acid composition suggests that they arise from specific enzymatic, rather than chemical, degradation of the parent cardiolipin. The overall pattern of mitochondrial fatty acids recorded here is similar to that reported by Fleischer and Rouser (16). The presence of large amounts of linoleic acid in cardiolipin and arachidonic acid in phosphatidyl ethanolamine is confirmed. A high percentage of saturated fatty acids (65 percent) and a sizable amount of arachidonic acid in mitochondrial phosphatidyl inositol is reported here for the first time.

Results reported herein suggest that cardiolipin is bound to cytochrome oxidase by both non-polar and ionic bonds, since it can be completely removed only by treatment with ionic reagents together with detergent or solvents. They further indicate preferential association of specific lipids with enzymes involved in mitochondrial electron transport.

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