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# TIGHTLY BOUND CARDIOLIPIN IN CYTOCHROME OXIDASE

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SUMMARY

Tightly bound cardiolipin has been found in cytochrome oxidase. The molar ratio of bound cardiolipin to cytochrome oxidase approaches 1:1. The tightly bound cardiolipin cannot be removed by many solvents which extract loosely bound lipids, but can be extracted with alkaline chloroform–methanol. Phospholipase A does not hydrolyze the bound lipid. After partial removal by repeated cholate–$(NH_4)_2SO_4$ treatment maximum activity can be restored in the oxidase by cardiolipin but not by detergents. The bound cardiolipin has a fatty acid composition similar to the bulk mitochondrial cardiolipin but small amounts of three other phospholipid fractions with a high level of saturated fatty acid are also found to be closely associated with the oxidase.

## INTRODUCTION

Several preparations of purified cytochrome oxidase have been reported with diverse phospholipid content[1–8]. Quite often, these studies have been aimed at ascertaining the possible role of phospholipid on function and structure of this enzyme. FLEISCHER *et al.*[9] and MCCONNEL *et al.*[10] have reported preparations of this enzyme having a phospholipid content equivalent to intact mitochondria. FLEISCHER *et al.*[9] recorded a higher percentage of diphosphatidyl glycerol (cardiolipin) in the enzyme as compared to mitochondria and other submitochondrial particles. On the other hand, MORRISON *et al.*[11] reported a highly purified preparation of cytochrome oxidase from pig heart mitochondria, using anionic detergents and $(NH_4)_2SO_4$ fractionation, which had less than 1% phospholipid. Although this lipid-depleted preparation was not very active, it could be reactivated by adding either phospholipids or detergents such as Emasol or Tween 80. However, the optimal activity reported by these authors is low in comparison to other reported values[5,12] and this has been attributed to presence of cholate in the preparation[8]. Another set of "lipid-free" preparations have been reported by JACOBS *et al.*[5] and SUN *et al.*[12] using the non-ionic detergents Triton X-100 and X-114. These preparations are not very active but addition of phospholipids or detergents cause much greater activation than reported by HORIE AND MORRISON[8]. A significant feature of this "lipid-free" oxidase is the presence of residual phospholipid, mainly comprising cardiolipin, which ranges from 1.5 to 1.8 $\mu$g phosphorus per mg protein and cannot be completely removed from the enzyme even after extraction with polar solvents like chloroform–methanol (2:1, by vol.)[13]. The present study deals with

complete characterization of this tightly bound phospholipid together with its fatty acid composition compared to that of mitochondrial lipids. Attempts have been made to remove a part of this lipid by detergent (ionic) and salt treatment and the resultant failure of detergents to restore activity together with better activation by phospholipids; cardiolipin in particular, develops a case for an essential role of this tightly bound cardiolipid in the function of this enzyme. Preliminary reports on the role of this tightly bound cardiolipin in cytochrome oxidase have been published[13,14].

## METHODS AND MATERIALS

Beef heart mitochondria were prepared according to method of LÖW AND VALLIN[15]. Methods for preparation of "lipid-free" cytochrome oxidase by Triton X-100 and X-114 treatments have been described earlier[16]. Phospholipid micelles of purified phosphatidyl ethanolamine, phosphatidyl choline or cardiolipin from beef heart mitochondria were prepared as reported earlier[17]. Phospholipase A from *Naja naja* venom was purified by heat treatment followed by chromatography over Sephadex G-75 according to CREMONA AND KEARNEY[18]. Membraneous cytochrome oxidase was prepared by deoxycholate–cholate treatment according to FOWLER *et al.*[7], followed by dialysis of the final product against 40 vol. of 0.05 M Tris–HCl (pH 7.8)[10]. "Reconstituted" membraneous cytochrome oxidase was prepared by sonicating "lipid-free" oxidase with excess phospholipid micelles in 0.01 M Tris–HCl (pH 7.4) (30 $\mu$g phosphorus per mg cytochrome oxidase protein) in a stainless steel cup submerged in an ice-salt bath for five 1-min periods. The sonicate was centrifuged at 105000 $\times$ *g* for 30 min and "membraneous oxidase" was collected as a green colored pellet. The pellet was resuspended in Tris–HCl (0.01 M, pH 7.4) and centrifuged at 105000 $\times$ *g* for 30 min. The operation was repeated 3 times to remove any trace of unbound phospholipid in the supernatant.

Phospholipase A digestions were carried out at 30° in 0.06 M Tris–HCl (pH 7.4) without adding exogenous $Ca^{2+}$ and phospholipid analysis of the digest was carried out as reported earlier[17]. Protein was estimated according to procedures outlined by YONITANI[19] and also by the method of LOWRY *et al.*[20]. Phosphorus was estimated by the method of CHEN *et al.*[21]. Cytochrome oxidase was assayed as reported earlier by CHUANG *et al.*[16].

Solvent extraction of cytochrome oxidase was carried out in the following manner: Cytochrome oxidase in Tris–HCl (0.02 M Tris, pH 7.4) was mixed with 20 vol. of the solvent and the mixture was shaken vigorously for 3 min. The solvent layer was separated by centrifuging in a clinical centrifuge and was carefully drained off. Four such extractions were carried out and the residual oxidase was analyzed for phosphorus content.

*Cholate–$(NH_4)_2SO_4$ fractionations*[8]

Saturated neutral $(NH_4)_2SO_4$ (pH 7.2) was added to "lipid-free" cytochrome oxidase in 0.02 M Tris–HCl (pH 7.4) (protein content 10 mg/ml) to a final concentration of 55 % saturation of $(NH_4)_2SO_4$ and cytochrome oxidase was obtained as a green oily pellet on centrifuging at 11000 $\times$ *g* for 15 min. The pellet was suspended in 0.1 M Tris–HCl (pH 7.8) containing 11.3 % $(NH_4)_2SO_4$ and 2.0 % potassium cholate, hereafter designated as Tris–cholate–$(NH_4)_2SO_4$ buffer. Final concentration was adjusted

to 10 mg protein per ml and the suspension was centrifuged at 15000 × *g* for 10 min. The pellet was discarded and $(NH_4)_2SO_4$ concentration of the supernatant was adjusted to 27 % saturation and centrifuged at 11000 × *g* for 10 min. The pellet was again discarded and $(NH_4)_2SO_4$ concentration of the supernatant was adjusted to 35 % saturation. Centrifugation at 15000 × *g* for 15 min yielded a green pellet containing cytochrome oxidase. The pellet was suspended in Tris-cholate-$(NH_4)_2SO_4$ buffer and protein concentration was adjusted to 10 mg protein per ml. 4 mg sodium dithionite per 10 ml were added and the $(NH_4)_2SO_4$ concentration was raised to 27 % saturation by adding solid $(NH_4)_2SO_4$. After stirring for 10 min it was centrifuged at 15000 × *g* for 15 min. The pellet was discarded and the $(NH_4)_2SO_4$ concentration of the supernatant was adjusted to 35 % saturation, the enzyme was obtained as a pellet on centrifuging at 15000 × *g* for 15 min. Enzyme was resuspended in Tris–HCl–cholate-$(NH_4)_2SO_4$ buffer to yield a protein concentration of 10 mg/ml and the $(NH_4)_2SO_4$ concentration was raised to 39 % saturation by adding solid $(NH_4)_2SO_4$, the enzyme was again obtained as a pellet by centrifugation at 15000 × *g* for 15 min. Enzyme was resuspended in Tris–HCl–cholate-$(NH_4)_2SO_4$ buffer and reprecipitated by raising $(NH_4)_2SO_4$ concentration to 35 % saturation. Twelve more such precipitations were carried out and the final pellet was resuspended in Tris–HCl (0.02 M, pH 7.4). Phosphorus content of different fractions was monitored throughout the isolation procedure.

*Phospholipid and fatty acid analysis*

Lipids were recovered from mitochondrial and cytochrome oxidase preparations by extracting twice with chloroform–methanol (2:1, by vol.), once with ethanol–diethyl ether (1:1, by vol.) and finally twice with chloroform–methanol–aqueous ammonia (7:1:5, v/v/ %). No phosphorus was detected in cytochrome oxidase residues after this extraction procedure. The combined extracts were evaporated *in vacuo* without heating, redissolved in chloroform–methanol and washed to remove non-lipid contaminants[22]. Lipids were stored in a $N_2$ atmosphere at —20° until analysis.

Polar lipids were resolved by two-dimensional thin-layer chromatography on 500-$\mu$ layers of silica gel HR in the solvent systems described by PARSONS AND PATTON[23]. The identity of components separated was verified by co-chromatography with authentic reference compounds (Applied Science, State College, Pa). Phospholipid distribution was determined by phosphorus analysis of components recovered from thin-layer chromatograms[24].

For fatty acid analysis lipid components were localized on chromatograms by spraying with 0.2 % 2,7-dichlorofluorescein in methanol, recovered, methylated and analyzed as described previously[25]. To prevent autoxidation, thin-layer plates were dried in an atmosphere of pure $N_2$. Identification of fatty acid methyl esters was accomplished by graphic plotting of their retention times in comparison to those of known methyl esters according to ACKMAN AND BURGHER[26]. Identity of unsaturated acids was further verified by analysis of methyl esters before and after hydrogenation in hexane over platinum oxide catalyst.

## RESULTS

Initially the phospholipid composition of intact beef heart mitochondria was reexamined (Table I). Besides the constituents reported in the table, traces of lyso-

TABLE I

PHOSPHOLIPID CONTENT OF MITOCHONDRIA AND DIFFERENT CYTOCHROME OXIDASE PREPARATIONS

| *Preparation* | *Total phosphorus (μg/mg protein)* | *Individual phospholipids (%) of total phospholipid phosphorus* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *PE* | *PC* | *DPG* | *PI* | *LPC* | *SP* | *Others* |
| Total mitochondria | 19.5 | 29.8 | 37.8 | 18.2 | 5.4 | 2.8 | 3.0 | 3.0 |
| "Lipid-free" cytochrome oxidase | 1.59 | 0 | 0 | 73.0 | 0 | 0 | 0 | 27.0 |
| Membraneous oxidase * (Triton) | 11.8 | 13.4 | 30.1 | 50.4 | — | — | — | 6.0 |
| Membraneous oxidase ** (cholate–deoxycholate) | 15.7 | 30.6 | 32.0 | 30.0 | — | — | — | 7.0 |
| "Reconstituted" membraneous oxidases *** | 20.0 | 30.0 | 34.0 | 30.0 | — | — | — | 6.1 |

Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; DPG, diphosphatidyl glycerol (cardiolipin); PI, phosphatidyl inositol; LPC, lysophosphatidyl choline; SP, sphingomyelin.

* Green fraction (crude cytochrome oxidase) obtained by Triton X-114 (2.6 mg/mg protein) and KCl (0.2 M) split.

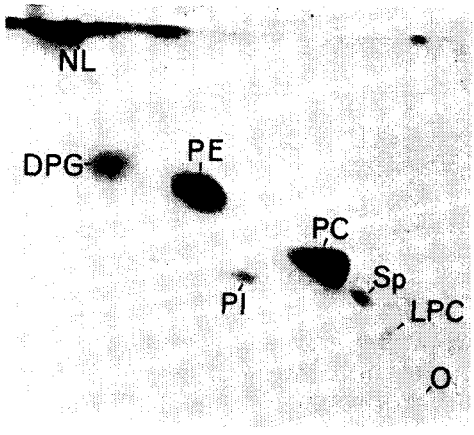
** Prepared by method of FOWLER *et al.*[7].

*** Obtained by sonicating (3 × 5 min) the "lipid-free" oxidase with micelles of total mitochondrial phospholipid in 0.01 M Tris–HCl (pH 7.4) in excess and centrifuging the membraneous pellet (105000 × *g*, 30 min). Unbound phospholipid removed by washing the pellet repeatedly with Tris–HCl (0.01 M, pH 7.4).

phosphatidyl ethanolamine were also detected. While the overall pattern of the results given here is similar to those reported by FLEISCHER AND ROUSER[27], the total percentage of three major phospholipids, *viz.* lecithin, phosphatidyl ethanolamine and cardiolipin, is only 85 % as compared to the 96 % reported by FLEISCHER AND ROUSER[27]. The presence of phosphatidyl inositol, sphingomyelin and lysophosphatidyl choline is revealed in well-resolved spots on the two-dimensional thin-layer plates (Fig. 1). Total phospholipid content of different preparations of beef heart mitochondria were in the range of 17–18 μg phosphorus per mg protein and all of this could be extracted by chloroform–methanol (2:1, by vol.) followed by chloroform–methanol–$NH_4OH$ (100:50:1.5, by vol.).

Phospholipid content and the composition of the phospholipids in various preparations of cytochrome oxidase are also given in Table I. Analysis of at least 50 different preparations of "lipid-free" cytochrome oxidase showed a phospholipid phosphorus content ranging from 1.5 to 1.8 μg phosphorus per mg protein. However, membranous cytochrome oxidase obtained by using the Triton–KCl red-green split in the procedural step prior to DEAE-cellulose column chromatography had a phospholipid content of 6–12 μg phosphorus per mg protein. Enzyme prepared by the method of FOWLER *et al.*[7] and also the reconstituted membraneous cytochrome oxidase obtained by sonicating the "lipid-free" enzyme with micelles of total mitochondrial phospholipid had phospholipid contents equivalent to 15–20 μg phosphorus per mg protein. The phospholipid composition of these different preparations shows a definite preference of the enzyme for cardiolipin over other lipids. Approx. 73 % of the total lipid phosphorus extracted from "lipid-free" cytochrome oxidase was in cardiolipin. Both of the other mitochondrial phospholipids, phosphatidyl ethanolamine and phosphatidyl choline, were absent in this preparation and three uncharacterized constituents, $X_1$, $X_2$ and $X_3$, appear (Fig. 2). These latter compounds appear to be degradation prod-

ucts of parent cardiolipin. "Reconstituted" membraneous oxidase and also deoxycholate–cholate preparations (ref. 7) have similar lipid composition, with all the three major lipids being present in nearly equal amount. Since only 18 % of the mitochondrial phospholipid is cardiolipin, the enzyme appears to have a preference for cardiolipin over other lipids. This is further confirmed by the lipid composition of membrane cytochrome oxidase obtained by Triton treatment without DEAE-cellulose column chromatography. More than 50 % of the total phospholipid of this preparation, which has 6–12 $\mu$g phosphorus per mg protein, was cardiolipin. Thus increasing percentages of cardiolipin in the residual lipids during removal of phospholipids from the enzyme clearly indicate that at least a part of the cardiolipin is tightly bound to the enzyme.



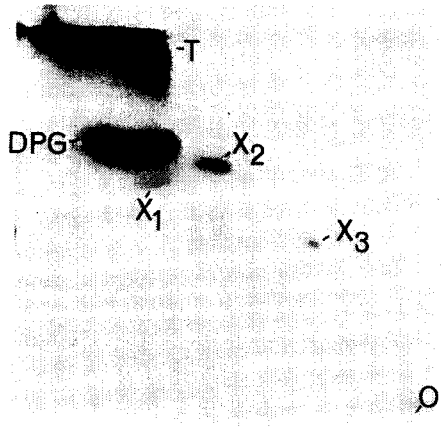




Fig. 1. Phospholipids of beef heart mitochondria. NL, neutral lipid; DPG, diphosphatidyl glycerol (cardiolipin); PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; Sp, sphingomyelin; LPC, lysophosphatidyl choline; O, origin.

Fig. 2. Phospholipids of "lipid-free" cytochrome oxidase. $X_1$, $X_2$ and $X_3$, uncharacterized phospholipids; T, Triton, DPG, diphosphatidyl glycerol (cardiolipin); O, origin.

Attempted extraction of this tightly bound cardiolipin from "lipid-free" cytochrome oxidase with different lipid solvents yielded only partial success. While isooctane, ether, *n*-hexane and acetone did not extract any of this tightly bound cardiolipin, chloroform–methanol (2:1, by vol.), a solvent nearly universally used for lipid extraction, removed only a part of this cardiolipin, the phosphorus content of the extracted enzyme varying from 0.8 to 0.9 $\mu$g phosphorus per mg protein (Table II). However, on extraction with chloroform–methanol–$NH_4OH$ 28 % (100:50:1.5, by vol.) all phospholipid was extracted and the residual enzyme contained no detectable phosphorus. Similar extractions were carried out on preparations according to FOWLER *et al.*[7] and results are given in Table II. With both Hatefi-type[7] preparations and "reconstituted" membraneous cytochrome oxidase preparations, prepared by interreaction of total mitochondrial lipids, complete extraction of phospholipids could not be achieved by acetone or chloroform–methanol and the level of the residual lipid after acetone extraction was close to that of "lipid-free" cytochrome oxidase. This residual lipid could be extracted by introducing ammonia in the solvent system and was found to consist mainly of cardiolipin.

The chloroform–methanol–ammonia extract of "lipid-free" enzyme showed that

cardiolipin represents 73 % of the phosphorus with the remainder in three other uncharacterized spots which were shown to be different from other lipid found in the untreated mitochondria. The uncharacterized spots were ninhydrin negative and showed different chromatographic properties than other major lipids of mitochondria on a two-dimensional thin-layer plate. The uncharacterized spots ($X_1$, $X_2$ and $X_3$, Fig. 2) had mobilities very similar to the degradation products prepared from a pure sample of cardiolipin by treatment with phospholipase A obtained from *N. naja* venom.

TABLE II

PHOSPHORUS CONTENT OF "LIPID-FREE" CYTOCHROME OXIDASE AND ITS EXTRACTION

| *Extraction solvent* | *Phosphorus content (μg phosphorus/mg protein)* | | |
|---|---|---|---|
| | *"Lipid-free" preparation** | *Deoxycholate preparation* | *Reconstituted membraneous oxidase* |
| None | 1.59 | 20.2 | 15.3 |
| Diethyl ether | 1.65 | 9.4 | 10.5 |
| *n*-Hexane | 1.60 | — | — |
| Isooctane | 1.68 | 16.0 | 13.1 |
| Acetone (dry) | 1.55 | 3.0 | 3.4 |
| Acetone (10% water)** | 0.72 | — | — |
| Acetone (10% water + $NH_4^+$)** | 0.57 | — | — |
| Chloroform–methanol (2:1, by vol.) | 0.80–0.62 | 1.8 | 1.0 |
| Chloroform–methanol–ammonia (100:50:2, by vol.) | 0.00–0.25 | 0.0 | 0.0 |
| Chloroform–methanol–12 M HCl (200:100:1, by vol.) | Protein solubilized | | |
| Pyridine | Protein solubilized | | |
| Triton X-114 + KCl (1 M) | 1.65 | | |
| Sodium dodecyl sulfate (0.05%) + $(NH_4)_2SO_4$ (10%) | 1.70 | | |
| Potassium cholate (2%) + $(NH_4)_2SO_4$ (11.3%)* | 0.93 | | |

* 14 fractionations, *cf.* HORIE AND MORRISON[8] and METHODS AND MATERIALS.
** Procedure of FLEISCHER *et al.*[29].

However, identification of these lipid materials has not been completed. In an attempt to establish the specificity in the fatty acid composition of these tightly bound phospholipids, the fatty acid composition of tightly bound cardiolipin and major uncharacterized products $X_1$ *plus* $X_2$ was studied along with the fatty acid composition of phospholipids of mitochondria. The fatty acid composition (Table III) suggests that, apart from a slightly higher level of saturated acids in the oxidase-bound cardiolipin, there is no significant difference in the fatty acid patterns of mitochondrial and cytochrome oxidase cardiolipin. However, the uncharacterized constituents $X_1$ *plus* $X_2$ have significantly different fatty acid compositions in comparison with cytochrome oxidase cardiolipin. High saturated acid content of these constituents suggests that if they arise by degradation of cardiolipin they must be derived by specific enzymatic breakdown rather than chemical degradation. These studies confirm the presence of a high level of unsaturated fatty acids in cardiolipin and in addition reveal a large content of stearic and arachidonic acids in mitochondrial phosphatidyl inositol.

TABLE III

FATTY ACID COMPOSITION OF BEEF HEART MITOCHONDRIA AND "LIPID-FREE" CYTOCHROME OXIDASE PHOSPHOLIPID (%)

| *Acid* | *Beef heart mitochondria* | | | | *Cytochrome oxidase* | |
|---|---|---|---|---|---|---|
| | *Cardiolipin* | *Phosphatidyl choline* | *Phosphatidyl ethanolamine* | *Phosphatidyl inositol* | *Cardiolipin* | $X_1 + X_2$ |
| 16:0 | 1.7 | 28.5 | 4.9 | 7.0 | 2.9 | 30.7 |
| 16:1 | 2.3 | — | — | — | 2.6 | — |
| 17:0 | — | — | — | 4.2 | — | — |
| 18:0 | 1.1 | 5.2 | 37.6 | 55.6 | 2.3 | 25.1 |
| 18:1 | 7.2 | 23.4 | 5.0 | 8.3 | 7.0 | 28.8 |
| 18:2 | 83.9 | 30.6 | 15.4 | 5.1 | 80.6 | 10.1 |
| 18:3 | 2.3 | 2.7 | 1.1 | 0.6 | 2.4 | |
| 20:3 | 0.9 | — | — | — | 0.7 | — |
| 20:4 | 0.7 | 4.9 | 28.2 | 14.1 | 1.5 | 3.1 |
| 24:0 | — | 0.9 | 4.1 | — | — | — |
| Unidentified* | | | | | | |
| Tr 3.05 | — | 3.3 | 2.2 | 5.1 | — | — |
| Tr 8.18 | — | — | 1.6 | | | |
| Tr 2.29 | — | — | — | — | — | 2.2 |

* Tr relative to methyl stearate.

*Activity of cytochrome oxidase*

Although the "lipid-free" enzyme is rather inactive it could be reactivated on addition of phospholipids and certain detergents. Whereas the addition of total mitochondrial phospholipids in micellar form activate the enzyme much more than does addition of deoxycholate, sodium dodecyl sulfate or Triton, Emasol and Tween 80 activate this enzyme to the same extent as phospholipids. Lysophosphatides also activate the enzyme but much less than intact phosphatides. Among the purified lipids of mitochondria, cardiolipin was found to be most active and the activity induced in the enzyme by cardiolipin was almost twice that induced by lecithin and 3 times that by phosphatidyl ethanolamine. However, the activation by cardiolipin was of the same order as by total lipids of mitochondria or Emasol. The low activity of cytochrome oxidase noted by HORIE AND MORRISON[8] in their more lipid-deficient enzyme led us to attempt a removal of tightly bound cardiolipin without destroying the enzyme. The cholate-$(NH_4)_2SO_4$ procedure of HORIE AND MORRISON[8] was applied to determine if extraction of tightly bound cardiolipin was possible. Fig. 3 shows the decrease of phosphorus level of the enzyme with number of cholate–$(NH_4)_2SO_4$ treatments. As many as fourteen repetitions removed only a part of this lipid and decrease of lipid with further fractionations appeared to level off. After fourteen fractionations the phosphorus level of the enzyme was 0.93 $\mu$g phosphorus per mg protein. As expected, the remaining phospholipid could only be extracted by chloroform–methanol–ammonia and it was shown to be comprised solely of cardiolipin along with uncharacterized constituents $X_1$, $X_2$ and $X_3$ (Fig. 2). This partially extracted "lipid-free" enzyme (by cholate–$(NH_4)_2SO_4$) was then tested for reactivation by phospholipids and detergents. While the initial activity of the enzyme rose from 0.9 to 27.9 on addition of cardiolipin, Emasol and Tween 80 increased activity only to 10.1 (Table IV). Activa-

tion of enzyme by total phospholipids and lecithin was of the same order and less than that by cardiolipin. Phosphatidyl ethanolamine was found to be the poorest activator of all the phospholipids and detergents evaluated. Lower activities of the cholate–$(NH_4)_2SO_4$-extracted enzyme in comparison with original "lipid-free" enzyme can be attributed to inhibition by cholate which is used during fractionation. However, results described here clearly indicate that Emasol or other detergents cannot replace the tightly bound lipid, and that residual cardiolipin is essential for activity of the enzyme. Although the treatment with cholate–$(NH_4)_2SO_4$ did not effect complete removal of this lipid, removal of a portion of this tightly bound cardiolipin and poor reactivation of the extracted enzyme by Emasol strongly suggest that this tightly bound cardiolipin plays a significant role in maintaining the function of passive "lipid-free" cytochrome oxidase. Low activities of pig heart cytochrome oxidase reported by HORIE AND MORRISON[8] could also be explained on the basis of loss of a major part of

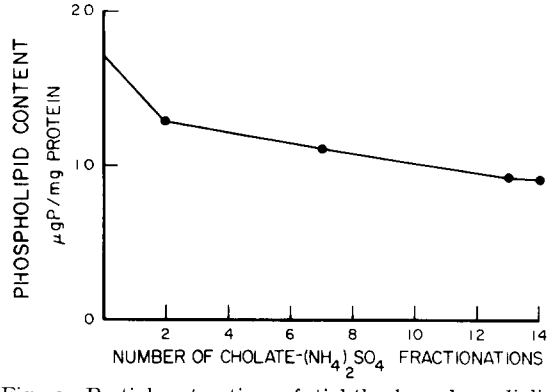



Fig. 3. Partial extraction of tightly bound cardiolipin of "lipid-free" oxidase by repeated $(NH_4)_2SO_4$–cholate treatment.

TABLE IV

RESTORATION OF ACTIVITY * OF "LIPID-FREE" CYTOCHROME OXIDASE BY LIPIDS AND DETERGENTS

| *Treatment* | *"Lipid-free" Triton preparation (1.6 μg phosphorus/mg protein)* | *"Lipid-free" Triton preparation after fractionation with cholate–$(NH_4)_2SO_4$ (0.93 μg phosphorus/mg protein)* | *Earlier study*[8] *(0.2–0.3 μg phosphorus/mg)* |
|---|---|---|---|
| None | 3.6 | 0.9 | |
| + Beef heart mitochondria ** phospholipids | 36.0 | 19.1 | |
| + Cardiolipin ** | 37.9 | 27.9 | |
| + Lecithin ** | 18.7 | 20.3 | |
| + Phosphatidyl ethanolamine ** | 12.4 | 2.2 | |
| + Emasol *** | 36.0 | 10.1 | 8.25 |

* Expressed as μmoles $O_2$ per min per mg protein.

** Enzyme was preincubated with 50 μg phosphorus of phospholipids per mg of enzyme protein before being diluted for assay.

*** 1% of Emasol-1130 in assay medium.

TABLE V

DIGESTION OF CYTOCHROME OXIDASE PHOSPHOLIPIDS BY PHOSPHOLIPASE A *

| *Preparation used for digestion* | % *Individual phospholipids cleaved* | | |
|---|---|---|---|
| | *Phosphatidyl ethanolamine* | *Phosphatidyl choline* | *Diphosphatidyl glycerol* |
| 1. Total mitochondrial lipids ** | 100 | 100 | >90 |
| 2. Hatefi-type[7] oxidase *** | 100 | 70–80 | 20–25 |
| 3. "Reconstituted" membraneous oxidase | 100 | 70–80 | 20–25 |
| 4. "Lipid-free" oxidase | — | — | Nil |
| 5. "Lipid-free" oxidase + sodium dodecyl sulfate (0.05 %) | — | — | Nil |
| 6. "Lipid-free" oxidase + $\Delta H$ (60°, 10 min) | — | — | Nil |

* 20 $\mu$g phospholipase A per mg protein, 30°, 60 min; 0.06 M Tris–HCl (pH 7.4).
** 30 $\mu$g phospholipase per mg phospholipid, 30°, 60 min; 0.06 M Tris–HCl (pH 7.4).
*** Deoxycholate–cholate preparation (ref. 7).

tightly bound lipid during their repeated fractionations. Attempts to extract the tightly bound lipid with other detergents, *e.g.* sodium dodecyl sulfate, were not successful since the phosphorus level of the extracted enzyme remained the same as that of original "lipid-free" enzyme.

The action of phospholipase A on the tightly bound cardiolipin was also tested. However, no digestion of "lipid-free" cytochrome oxidase cardiolipin was observed under conditions[17] which hydrolyze the phospholipids of mitochondria and lipid-rich cytochrome oxidase (Table V). The lipid remained unaffected by the lipase after treatment of the enzyme with sodium dodecyl sulfate or denaturation of enzyme by heat treatment. These experiments suggest that fatty acid moieties of the tightly bound lipid are not accessible to phospholipase A.

## DISCUSSION

Our results suggest a remarkably strong association between cardiolipin and cytochrome oxidase. Based on 4 % phosphorus in phospholipids and taking molecular weight of cytochrome oxidase to be 72000 (ref. 28), the molar ratio between oxidase and cardiolipin would be 1 : 1.3. However, if we include the uncharacterized constituents whether they represent endogenous elements or degradation products of tightly bound cardiolipin this ratio would be close to 1 : 2. The cardiolipin–enzyme complex though not active in the native state, can be activated fully on addition of phospholipids or detergents like Emasol and Tween 80. As discussed earlier[14] this complex is not in a membraneous state but takes part in membrane organization on addition of phospholipids. Even a partial removal of tightly bound cardiolipin results in some change in the enzyme so that it can not be fully activated on adding detergents. However, phospholipids, and cardiolipin in particular, activate the enzyme almost completely. From these results, one may envisage a dual role of phospholipids in the catalytic function of cytochrome oxidase. In the first place, 1–2 molecules of phospholipid are necessary to form a stable lipoprotein complex. Additional phospholipid is then required for optimum activity, probably by exposing more active sites of the enzyme to substrate mole-

cules. This view is substantiated by marked transition in the aggregation state of cytochrome oxidase with increasing phospholipid content reported earlier[14]. Certain detergents like Emasol or Tween 80 can replace phospholipid insofar as their dispersing role is concerned but cannot replace cardiolipin in the internal lipid–protein interaction. Thus 1–2 moles of cardiolipin per mole cytochrome oxidase is essential and the resultant enzyme–lipid complex can then be fully activated by dispersion or membrane formation with either phospholipids or detergents. Though we cannot define the nature of binding between the lipid and protein, our experiments do offer some significant insight. Since $NH_4OH$ is required for complete extraction of lipid with chloroform–methanol and since ionic detergent and salt treatments extract a portion of the cardiolipin, it appears that at least a part of it is bound electrostatically. On the other hand, the lack of effect of phospholipase A suggests that fatty acid chains of cardiolipin are well fortified and either the fatty chains or entire molecule of cardiolipin itself is buried within the hydrophobic region of the protein. Thus both ionic and non-polar bonding through unsaturated fatty acid residues are feasible. The present study calls for an effective method for complete removal of tightly bound lipid from cytochromes oxidase which will allow a more precise determination of the role which phospholipids play in the function of the enzyme.

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