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CARDIOLIPIN REQUIREMENT BY CYTOCHROME OXIDASE AND THE CATALYTIC ROLE OF PHOSPHOLIPID

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SUMMARY

An effective method is described for the almost complete removal of phospholipid from cytochrome oxidase, with a consequent parallel decrease in enzymic activity. Replenishment of lipid-depleted cytochrome oxidase with purified phospholipids indicates an absolute requirement of enzymic activity for cardiolipin. This catalytic role of cardiolipin is distinguishable from the more general effects of phospholipids and detergents on cytochrome oxidase activity.

INTRODUCTION

Depending on the method of isolation used, preparations of cytochrome oxidase (EC 1.9.3.1) may contain from 1 to 20% by wt. of phospholipid (1). Numerous investigations have purported to show a catalytic requirement in this enzyme for phospholipids, either individual or mixed, or for some dispersing detergent (2, and references therein). In a recent report from this laboratory (3) we had studied the effects of various phospholipids and detergents on cytochrome oxidase activity and we came to the general conclusion that most phospholipids, alone or as mixtures, were effective in activating the enzyme. These studies demonstrated that lipid-depleted preparations of cytochrome oxidase, possessing minimal respiratory activity, could be reactivated by interaction with phospholipids or detergents, and in particular by lysolecithin; thus there was no absolute requirement for phospholipids per se in such cytochrome oxidase preparations. However, an important feature of so-called "lipid-free" preparations of cytochrome oxidase (4,5) is the presence of residual

In order to avoid confusion in the text we shall characterize the phospholipid content by wt. of the different cytochrome oxidase preparations by the notation C.O.$_{x\%}$.

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phospholipid, mainly comprising cardiolipin (diphosphatidyl glycerol), that cannot be completely removed from the enzyme even after extraction with solvents such as chloroform/methanol [2:1 by vol.] (6).

These findings as well as our own studies on the requirements for activation of cytochrome oxidase (3) have led us to the presumption of two roles for phospholipid in cytochrome oxidase. One effect of phospholipid, particularly evident in "low-lipid" preparations of cytochrome oxidase, is that of solubilizing or plasticizing the enzyme into a state of order optimal for enzymic activity and interaction with cytochrome c; under such conditions the effects of phospholipids on enzymic activity are likely very similar in action to that of detergents. In contrast to this purely dispersive effect of phospholipids, we provide evidence in the present paper for an absolute and specific catalytic requirement of cytochrome oxidase for cardiolipin. It has been shown (7) that cardiolipin remains firmly bound to cytochrome oxidase even after extraction methods have removed the majority of bound phospholipids. Until more recently it has not been possible to remove these last molecules of cardiolipin from the enzyme without causing denaturation and irreversible loss of activity, and so the absolute catalytic requirement for cardiolipin could not have been shown (only the more general requirement for phospholipids and detergents). These difficulties have been circumvented by the method presently described that allows for the almost complete phospholipid depletion and consequent demonstration that cardiolipin is specifically required for the cytochrome oxidase catalyzed reduction of molecular oxygen. In this report we provide evidence that phospholipid in addition to its purely dispersive effect has also a catalytic role. Whereas phospholipids generally can satisfy the dispersive role, only cardiolipin specifically can satisfy the catalytic role.

MATERIALS AND METHODS

Triton X-100 was purchased from Rohm and Haas, glycerol (99.5% pure) from Fisher Scientific Co., and cholic acid was recrystallized twice from ethanol before use. All phospholipids were obtained in purest form from Sigma Chemical Co. except for asolectin (95% pure from Associated Concentrates) and whole mitochondrial phospholipid which was prepared from beef heart mitochondria by the method of Fleischer et al. (8).

Cytochrome oxidase was prepared by the method of Fry et al. (9). Protein was determined by the biuret method (10) using bovine serum albumin as standard, and phospholipid content was calculated from an analysis of total phosphorus (11) and assuming 4% phosphorus content in phospholipids. Identification of extracted phospholipids was made by bi-dimensional thin-layer chromatography on 0.25 mm thick Silicagel 60 on glass plates according to Awasthi et al. (6).

Cytochrome oxidase activity was assayed by measurement of oxygen uptake at 38°C using a Clark-type oxygen electrode; 7 ml of 50 mM potassium phosphate buffer, pH 7.0, contained 25 mM ascorbate, 70 μM cytochrome c and 0.7 mg/ml of lysolecithin

The phospholipid content of cytochrome oxidase was depleted to varying extents by the following procedure. Cytochrome oxidase, as prepared, was first solubilized to about 10 mg. protein/ml in a solution containing 5% (v/v) Triton X-100, 20% (v/v) glycerol, and 50 mM Tris-HCl, pH 7.0. Up to 7 ml (about 60 mgm protein) of this solution was applied to an 4 x 18 cm Sephadex LH-20 column and eluted with a solution of 5% (v/v) Triton X-100, 50 mM in Tris-HCl, pH 7.0, with a flow rate of 0.9 ml per minute. The eluted enzyme was collected (diluted some 5-fold) and precipitated by addition of a saturated solution of ammonium sulfate to 25% saturation. Following centrifugation for 5 min. at 2,000 rpm, the precipitated enzyme was collected as an oily, floating layer. For more complete phospholipid removal this procedure was repeated up to four times, each time dissolving the enzyme in Triton X-100/glycerol before column application. Finally prepared samples of enzyme were dissolved in 50 mM Tris-HCl, pH 7.0, containing 1% (v/v) cholate, to about 1 mg. protein/ml. All the above operations were carried out at 4°C.

Chloroform or ethanolic solutions of phospholipids were evaporated to dryness under vacuum and resuspended to 25 mg/ml in 50 mM Tris-HCl, pH 7.0, containing 1% (v/v) cholate. These suspensions were maintained under nitrogen, sonicated (bath-type sonifyer) until clarified, and stored on ice until required.

RESULTS

Cytochrome oxidase, prepared by the method of Fry et al. (9) contains about 6% by wt. of phospholipid. This level of phospholipid can be substantially reduced by some 50% with simple passage through an LH-20 column. However, in order to further reduce the level of phospholipid it is necessary to precipitate the enzyme from Triton X-100 solution with ammonium sulfate and repeat the column fractionation procedure. The results of such procedures are tabulated in Table I. Up to four column fractionations were successful in reducing the phospholipid content of cytochrome oxidase to less than 0.5% by wt. The phospholipid content could be reduced from 6.3% to 2.8% by wt. with almost no loss of enzymic activity, and further to 1.4% by wt. while still retaining significant activity. Only when the phospholipid content was reduced to less than 1.4% by wt. did the enzymic activity decrease significantly, and with a lowest phospholipid content of about 0.4% by wt. catalytic activity also reached its lowest value. Depletion of the

TABLE I

PHOSPHOLIPID DEPLETION OF CYTOCHROME OXIDASE AND CORRELATION WITH ENZYMIC ACTIVITY

No. of passages through LH-20 column	% by wt. phospholipid in cyt. oxidase		% phospholipid remaining		Activity (μmol O/min/mg prot.)		% of original activity	
None	6.3	6.2	-	-	37	35	-	-
One[a]	2.8	2.6	-	-	31	31	-	-
One	1.9	1.7	100	100	26	25	100	100
Two	1.1	0.9	58	53	16	13	59	52
Three	0.7	0.6	37	38	9.5	9	36	38
Four	0.4	0.3	21	22	5	5	19	20

Results are given from two separate experiments on different preparations of cytochrome oxidase.

[a]Cytochrome oxidase was assayed directly from solution in 5% Triton X-100 without precipitation by ammonium sulfate. All other assays were made after subsequent precipitation by ammonium sulfate followed by solubilization in 5% Triton X-100/20% glycerol.

phospholipid content of cytochrome oxidase below 1.4% by wt. was almost exactly paralleled by the decrease in enzymic activity (Table I).

When a $C.O._{2.8\%}$ preparation of cytochrome oxidase (achieved by one passage through LH-20) was thoroughly extracted with alkaline chloroform/methanol, the only detectable phospholipid extracted was cardiolipin (Fig. 1.)

Using a phospholipid depleted preparation of cytochrome oxidase (less than 0.5% by wt.), purified phospholipids were recombined with the enzyme in cholate solution and their effect on enzymic activity studied (Table II). With the exception of cardiolipin and whole mitochondrial phospholipid, all phospholipids tested were ineffective in stimulating enzymic activity, measured in the presence of lysolecithin. Cardiolipin stimulated enzymic activity to almost 85% of the original activity of a $C.O._{6\%}$ (original cytochrome oxidase as prepared) and to almost 95% of the original activity of a $C.O._{2.8\%}$ preparation (prepared by one

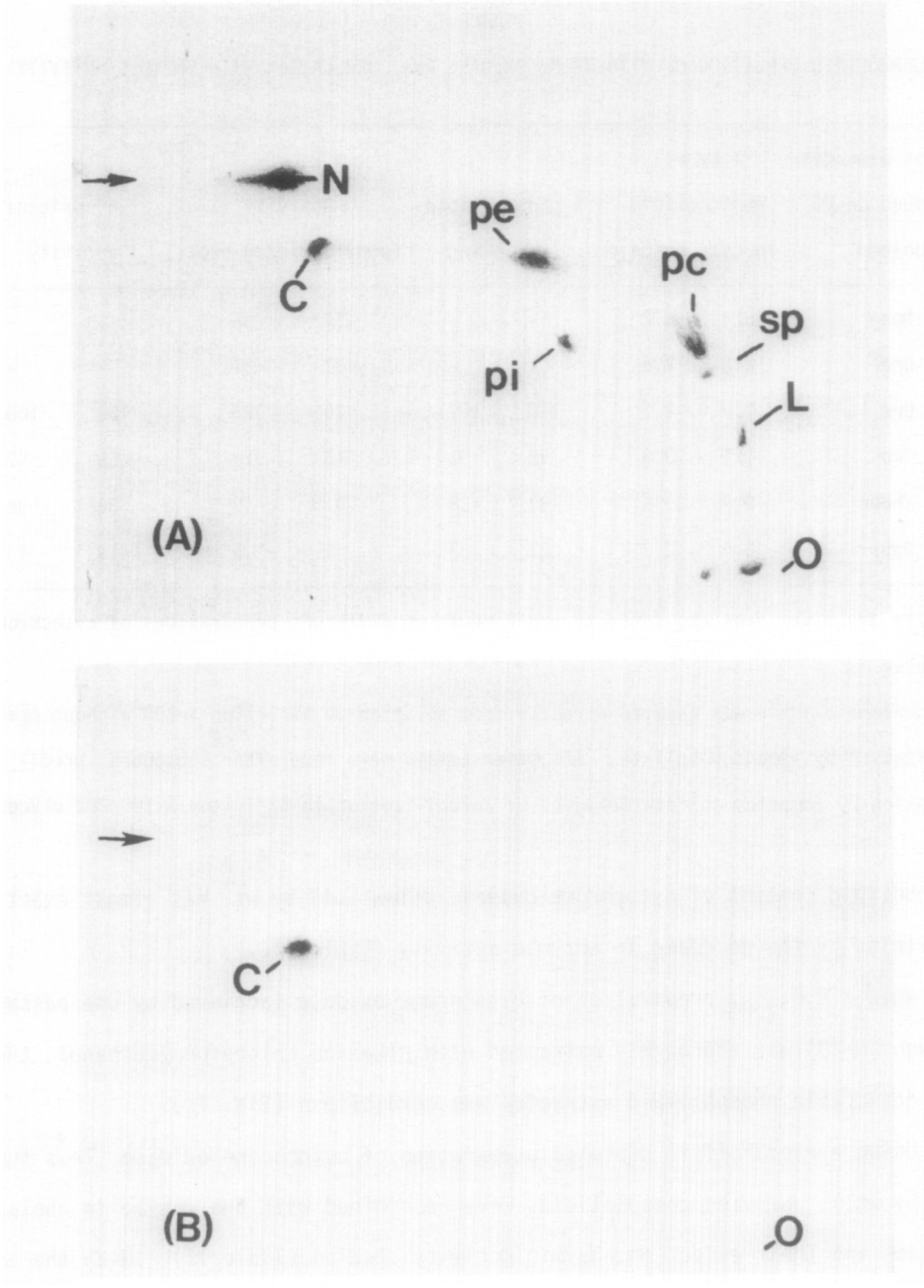

Fig. 1. Bi-dimensional thin-layer chromatography of a phospholipid extract from cytochrome oxidase. A low lipid preparation of cytochrome oxidase (about 2.8% by wt., from one passage through an LH-20 column) was extracted for 4 hr. at room temperature with chloroform:methanol:2M KOH (200:100:3). Phospholipid standards used on plate A were C = Cardiolipin, N = Neutral mitochondrial phospholipid, L = Lysolecithin, sp = sphingomyelin, pc = phosphatidylcholine, pe = phosphatidylethanolamine, and pi = phosphatidylinositol. Plate B is the cytochrome oxidase phospholipid chromatogram. O = origin. Solvent systems used were 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 were visualized by charring with 50% aqueous H_2SO_4.

TABLE II

REACTIVATION OF PHOSPHOLIPID DEPLETED CYTOCHROME OXIDASE

Phospholipid added	Activity (μmol O/min/mg prot.)
None	6
Cardiolipin	29.5
Mitochondrial phospholipid	13.2
Phosphatidylcholine	6.2
Phosphatidylethanolamine	6.0
Phosphatidylserine	5.9
Phosphatidylinositol	6.2
Phosphatidic acid	6.4
Asolectin	5.8

Phospholipid depleted cytochrome oxidase (0.4% by wt. phospholipid) was interacted with purified phospholipids (20:1, protein to phospholipid wt. ratio) in 50 mM Tris-HCl, pH 7.0, containing 1% (v/v) cholate. Samples were incubated at 38°C for 20 min. followed by 60 min. on ice before assay.

passage through LH-20; see Table I). Mitochondrial phospholipid stimulated enzymic activity to some 42% of the activity of a $C.O._{2.8\%}$ preparation.

Following interaction with purified phospholipids, cytochrome oxidase was precipitated from cholate solution with ammonium sulfate (to 25% saturation) and analysed to determine to extent of replenishment by various phospholipids. Table III provides the data that shows that almost the same degree of rebinding of phospholipids by lipid-depleted enzyme ($C.O._{0.5\%}$) was achieved for all the phospholipids tested. An average of some 1.6% by wt. of phospholipid was rebound by the lipid-depleted enzyme under these conditions.

DISCUSSION

The methodology examined in the present paper provides a convenient and effective means of almost completely removing all bound phospholipid from cyto-

TABLE III

REPLENISHMENT OF PHOSPHOLIPID TO PHOSPHOLIPID DEFICIENT CYTOCHROME OXIDASE

Phospholipid added	Final % by wt. phospholipid	Activity (μmol O/min/mg prot.)
None	0.4	5.2
Cardiolipin	2.4	28.7
Mitochondrial phospholipid	3.3	11.8
Phosphatidylcholine	1.3	5.2
Phosphatidylethanolamine	1.5	5.4
Phosphatidylserine	2.2	5.1
Phosphatidylinositol	1.5	5.2
Phosphatidic acid	1.8	5.9
Asolectin	2.1	5.5

Phospholipid depleted cytochrome oxidase was interacted with purified phospholipids according to the legend of Table II. Enzyme protein was precipitated by addition of ammonium sulfate to 25% saturation, resuspended in 50 mM Tris-HCl, pH 7.0, and precipitated once more with ammonium sulfate before analysis for phosphorus content.

chrome oxidase without denaturation of the enzyme. Indeed, preparations of lipid depleted enzyme can be stored in 5% Triton X-100/20% glycerol at 4°C for some days without any appreciable loss of recoverable activity. The use of Triton X-100 to delipidate beef-heart cytochrome oxidase has been successfully employed by Robinson (12) using a glycerol gradient technique. The present study has introduced the use of a Sephadex LH-20 column that increases the effectiveness of Triton X-100 as a delipidating agent.

This study confirms (12) the absolute requirement for cardiolipin in the cytochrome oxidase catalyzed reaction. Replenishment of phospholipid, and specifically cardiolipin, led to good recoveries of enzymic activity. It was

significant that although all phospholipids tested were found to rebind to the enzyme to about the same extent, only cardiolipin (and mitochondrial phospholipid containing cardiolipin) led to an effective regeneration of enzymic activity. This specific activation by cardiolipin is consistent with the sole presence of this phospholipid in low phospholipid preparations of cytochrome oxidase.

From these and recent studies (3) it is possible to distinguish between two roles of phospholipid in cytochrome oxidase. The enzymic activity of phospholipid-depleted preparations of cytochrome oxidase (of about 2% by wt. in phospholipid) is very low but can be restored through the addition of phospholipids or detergents (3). This activation effect is a general phenomenon and probably results from a physical effect on the state of aggregation of the enzyme. It is for this reason that we always include lysolecithin in the assay for cytochrome oxidase activity; this powerful activating agent (3) satisfies the requirement for correct dispersion of the enzyme while allowing for an evaluation of the catalytic role of cardiolipin in the enzyme complex. In low lipid preparations of cytochrome oxidase (less than 2% by wt. phospholipid), approximately two molecules of cardiolipin per molecule of cytochrome oxidase fulfill a necessary catalytic role in the enzyme. Further removal of this tightly bound cardiolipin results in a parallel decrease in enzymic activity that can only be restored by replenishment with cardiolipin.

The demonstration of a catalytic requirement for cardiolipin in cytochrome oxidase raises obvious questions about the nature of the catalytic function. Cytochrome oxidase as isolated is a coupled system and cardiolipin undoubtedly plays a key role in the coupling process.

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