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LIPID-SUBSTITUTED CYTOCHROME OXIDASE: NO ABSOLUTE REQUIREMENT OF CARDIOLIPIN FOR ACTIVITY.*

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SUMMARY

The endogenous lipid of yeast cytochrome oxidase has been replaced by dimyristoyl phosphatidylcholine. Thin layer chromatography of the total lipid extract from the substituted enzyme revealed phosphatidylcholine only and no cardiolipin. Gas-liquid chromatography showed that >99% of the lipid chains derived from the substituted lipid, and that cardiolipin must be <0.03 mole/mole enzyme. The activity of the lipid-substituted enzyme was 10% of the original activity and increased to 47% by addition of dimyristoyl phosphatidylcholine. Thus there is no absolute requirement of cardiolipin for oxidative activity.

Cardiolipin (diphosphatidyl glycerol) comprises 18% of the total phospholipids in mitochondrial membranes from beef heart (1). A role for cardiolipin in mitochondrial function is clearly indicated. Early reports (2,3) showed that endogenous phospholipid remains bound to cytochrome oxidase even after extraction with acetone or following successive ammonium sulphate precipitations in the presence of cholate. Later more detailed studies (1,4,5) indicated that approximately 1-6 moles of cardiolipin per mole of beef heart cytochrome oxidase are tightly bound. Awasthi et al (1) further concluded that the bound cardiolipin is essential for cytochrome oxidase activity. The results of biophysical studies (6,7) have lead to the suggestion that the negatively charged head groups of cardiolipin may be involved in the binding of ferrocytochrome c to cytochrome oxidase. A possible objection to the experiments reported by Awasthi et al (1)

* Abbreviations used are: TLC, thin layer chromatography; GLC, gas-liquid chromatography; DMPC, dimyristoyl phosphatidylcholine.

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is that the cardiolipin might be trapped in protein aggregates during lipid depletion. We have employed a procedure whereby endogenous phospholipids can be replaced by a defined lipid and which therefore minimises the chance of protein aggregation. The results of TLC and GLC analyses on such defined complexes of yeast cytochrome oxidase with dimyristoyl phosphatidyl choline clearly show that no cardiolipin is present. Since the complexes have enzyme activity, it can be concluded that cardiolipin is not essential for the oxidative activity of yeast cytochrome oxidase.

MATERIALS AND METHODS

Dimyristoyl phosphatidyl choline (DMPC) was obtained from Fluka; cholic acid was purchased from Sigma and crystallised twice from ethanol/water before conversion to the sodium salt. Cardiolipin was isolated from yeast mitochondria as described by Virji and Knowles (8). Submitochondrial particles were prepared from baker's yeast as described by Tzagoloff (9). Cytochrome oxidase (E.C.1.9.3.1) was isolated according to Eytam and Schatz (10) and stored at -20°C in the presence of 1% Tween 80.

The endogenous lipids and detergents were exchanged for DMPC by the following procedure. Enzyme was incubated at 25°C for 1 hour in the presence of DMPC (1000x molar excess) and sodium cholate (final concentration 0.2%); the buffer used was tris (10mM, pH7.0), KCl (1.0M), sucrose (1%). The enzyme was then precipitated by the addition at 0°C of saturated ammonium sulphate solution to a final saturation of 35%, followed by centrifugation (45 mins at 105,000g). The resuspended pellet was subjected to a second DMPC exchange by exactly the same procedure.

The sample was prepared at the desired lipid/protein ratio by a third exchange in the presence of DMPC (1000x molar excess) and cholate (0.3% final concentration). After incubation at 25°C, the cytochrome oxidase was precipitated by addition of ammonium sulphate, and centrifuged. The pellet was resuspended in tris/KCl/sucrose buffer containing 0.3% cholate and divided into two aliquots to give the sample for study (SAMPLE I), and a control sample (SAMPLE II) to which yeast cardiolipin (1 mole/mole enzyme) was added. Finally, the samples were dialysed for 17 hours at 15°C against this same buffer containing Amberlite XAD-2 resin. Portions of the dialysed samples were extracted according to Awasthi et al (1) and the lipid extracts stored (briefly) at -20°C under nitrogen prior to TLC and GLC analysis.

Silica gel TLC was performed on freshly-coated microscope slides using the solvent system $CHCl_3$/MeOH/25% NH_4OH (65/30/3 v/v). The plates were developed using Dittmer and Lester spray (11), followed by charring. Samples for GLC were prepared by methanolysis of the extracted lipids using the procedure of Glass (12). GLC was carried out on a Perkin Elmer GCF7 apparatus using a column of ethylene glycol succinate.

Enzyme activities were determined on samples (III and IV) which were prepared by the same procedure used for samples I and II respectively; the dialysed samples and a sample of enzyme as isolated were incubated at 0°C for 16 hours in the presence of 0.5% cholate together with, in some cases, excess DMPC prior to assay according to Mason et al (13).

Table 1. Composition of the DMPC-exchanged cytochrome oxidase samples and of their lipid extracts

	SAMPLE I (no added cardiolipin)	SAMPLE II (with added cardiolipin)
Before lipid extraction		
Phosphate nM ml^{-1}	635	654
Phosphate/protein (moles/mole)	41	45
After lipid extraction		
Extracted lipid		
Phosphate nM ml^{-1}	592	650
Protein	undetectable	undetectable
Residue		
Phosphate nM ml^{-1}	14	8
Phosphate/protein (moles/mole)	0.6 - 1.2	0.3 - 1.1

Protein was determined by the method of Dulley and Grieve (14) and phosphate according to Eibl and Lands (15). Lipid/protein ratios were based on a molecular weight of 200,000 for cytochrome oxidase.

RESULTS

The results of protein and phosphate assays before and after lipid extraction are given in Table 1. It is clear that essentially all of the lipid has been removed by the solvent extraction; the lipid remaining with the protein after extraction amounted to less than 1.2 mole lipid phosphate/mole protein. Even if this residual phosphate were all cardiolipin it would represent no more than 0.3-0.6 mole cardiolipin/mole protein.

TLC analysis of sample I revealed only phosphatidylcholine, and in particular revealed no cardiolipin, even at high loading. Similar analysis of sample II readily revealed the presence of the added cardiolipin, thus

Table 2. Percentage composition of alkyl chains in the extracts of DMPC-exchanged cytochrome oxidase.

Lipid	C14;0	C16:0	C16:1	C18:1
SAMPLE I				
(no added cardiolipin)	94	4.9	-	0.2
SAMPLE II				
(added cardiolipin)	88.3	5.6	3.5	2.6
DMPC (ex Fluka)	97.5	2.5	-	-
yeast cardiolipin	3.1	1.0	47.6	48.3

demonstrating that 1 mole cardiolipin/mole cytochrome oxidase could be extracted and easily detected under the conditions used.

Table 2 presents the results of the GLC analysis. The data show that the alkyl chain composition of yeast cardiolipin is mainly C16:1 and C18:1. The chain composition of the extracted lipid from sample I indicates a >99% exchange by the C14:0 and C16:0 chains of the substituting DMPC. The composition of the remaining chains requires that cardiolipin be present at a level of less than 0.03 moles per mole of enzyme. Analysis of the lipid extract from sample II shows that C16:1 and C18:1 chains compose 6% of the total showing that the added cardiolipin can be extracted and readily detected by GLC.

Exactly similar TLC and GLC results were also obtained when the extraction was performed using acidic and/or neutral solvent extraction procedures (16) rather than the alkaline media of Awasthi *et al* (1).

The results of enzyme activity assays given in Table 3 clearly demonstrate that sample III has considerable activity. It can be seen that the DMPC exchanged enzyme in the presence of excess DMPC has 47%

Table 3. Enzyme activity of DMPC exchanged cytochrome oxidase.

SAMPLE	Lipid/protein (moles/mole)	Specific Activity (min^{-1} mg^{-1})	% isolated activity
Isolated enzyme	not determined	4280	100
Sample II (no added cardiolipin)	48		
After incubation with cholate		436	10.2
" " +DMPC		2030	47.4
Sample IV (with added cardiolipin)	66		
After incubation with cholate		385	9.0
" " +DMPC		1965	46.0

of the activity of isolated enzyme. Moreover, the activity of sample IV, which has associated cardiolipin, is within experimental error the same as sample III. It may be concluded that cardiolipin is not essential for the activity of yeast cytochrome oxidase.

DISCUSSION

The results show that yeast cytochrome oxidase complexes having no associated cardiolipin can be prepared by a procedure utilising lipid replacement rather than lipid depletion. Since these complexes have significant enzyme activity, it is clear that cardiolipin is not required for the activity of yeast cytochrome oxidase. The relatively low enzyme activity is due in part to the fact that the complexes have only 40-50 moles of lipid per mole of enzyme which is below the level necessary to provide the complete annulus required for enzyme stability (17). This low lipid/protein ratio was chosen in order to optimise conditions for

detection of possible residual cardiolipin. At higher lipid/protein ratios, complexes with full activity are obtained (17).

It is not valid to conclude from the present results with yeast cytochrome oxidase that cardiolipin is not essential for the activity of the beef heart enzyme though this possibility merits re-investigation. Also it should be stressed that the results do not exclude a specific role for cardiolipin associated with cytochrome oxidase in membrane preparations. This role might be concerned with coupled phosphorylation (18) or the vectorial arrangement of cytochrome oxidase in its lipid matrix (19).

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