Phospholipid requirements for the reconstitution of Complex-III vesicles exhibiting controlled electron transport

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Phospholipid requirements for the reconstitution of Complex-III vesicles exhibiting respiratory control (electron-transport control) were studied. Vesicles prepared from pure phosphatidylethanolamine gave maximal control ratios. Phosphatidylcholine alone did not support respiratory control, although these vesicles were capable of maintaining stable K^+ -diffusion gradients. Apparently Complex III cannot insert into a bilayer of phosphatidylcholine. Formation of mixed phosphatidylcholine/phosphatidylethanolamine (6:1, w/w) vesicles was sufficient, however, to allow Complex-III insertion and to restore respiratory control. Mixtures of acidic phospholipids with either phosphatidylethanolamine or phosphatidylcholine did not improve respiratory control over that obtained with pure phosphatidylethanolamine. Phosphatidylethanolamine from bovine heart mitochondria, soya beans or *Escherichia coli* was equally effective in reconstituting respiratory control, suggesting that the specificity is referable to the head group and not to the fatty-acid moiety.

Ubiquinone-cytochrome *c* oxidoreductase (Complex III) purified from mitochondria has previously been incorporated into phospholipid vesicles that exhibit controlled electron transport (Leung & Hinkle, 1975; Guerrieri & Nelson, 1975; Eytan *et al.*, 1977; Wielburski & Nelson, 1978). Such functional reconstitution has been obtained with widely varying lipid compositions. In the present paper, the phospholipid specificity has been studied. We find that, of the major phospholipid classes in mitochondria, phosphatidylethanolamine is specifically required for reconstituting Complex III into vesicles capable of respiratory control.

Methods

Materials

Complex III was isolated from bovine heart mitochondria by the method of Rieske *et al.* (1964). Mitochondrial lipids were prepared from bovine heart mitochondria (Blair, 1967; Bock & Fleischer, 1974) by extraction with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957; Rouser & Fleischer, 1967), and non-lipid material was removed by Sephadex column chromatography (Siakotos & Rouser, 1965). Silicic acid column chromatography was used to separate the neutral lipids from the mitochondrial phospholipids and to purify the component phos-

pholipids (Isaacson *et al.*, 1979). Soya-bean phospholipids were a gift from Dr. Hans Betzing and Dr. Hans Genthe of A. Natterman and Cie, G.m.b.H., Cologne, Germany. *Escherichia coli* phosphatidylcholine was a gift from Dr. John Law, Department of Biochemistry, University of Chicago, Chicago, IL, U.S.A. (L- α -Dimyristoyl)phosphatidylethanolamine was obtained from Calbiochem-Behring Corp., LaJolla, CA, U.S.A. All purified phospholipids were judged to be at least 99% pure on the basis of separation by t.l.c. and phosphorus analysis of the spots made visible by charring (Rouser *et al.*, 1970).

Reconstitution of Complex-III vesicles

Reconstitution experiments of Complex III were carried out in the cold-room in a multi-welled micro-dialyser capable of dialysing eight samples (Rydström & Fleischer, 1979). Phospholipid (10mg/ml) containing potassium cholate (6.6mg/ml), 50mM-Tris/HCl, pH 7.5, 100mM-KCl and 1mM-EDTA was sonicated at room temperature in a bath sonicator (Laboratory Supplies, Nickesville, NY, U.S.A.) until clear (1–3 min), under an atmosphere of N_2 . In most instances, samples (75 or 100 μ l) were placed into a well to which 3 μ l of Complex III (80 μ g of protein) was also added. Samples were dialysed for 12–16 h against 600 ml of

buffer containing 50 mM-Tris/HCl, pH 7.5, 100 mM-KCl and 1 mM-EDTA. Each reconstitution was run in duplicate. For the study in Fig. 1, the concentration of Complex III in the wells was varied. Protein was measured by the biuret method.

Enzyme assay

Duroquinol-cytochrome *c* reductase was measured at room temperature (22°C) as described previously (Nelson & Gellerfors, 1978). The 'respiratory control ratio' is the ratio of the rate of cytochrome *c* reduction in the presence of 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine ('FCCP') divided by the rate in the absence of uncoupler.

Results

Reconstitution of respiratory control with individual phospholipids

Respiratory control can be obtained by incorporating Complex III into liposomes of purified phosphatidylethanolamine from either bovine heart mitochondria (Table 1) or soya beans (Table 2). The respiratory control ratio obtained with phosphatidylethanolamine alone is comparable with that obtained with mitochondrial phospholipids, i.e. the mixture of phospholipids in the mitochondrial inner membrane. In contrast, respiratory control is not reconstituted with purified preparations of phosphatidylcholine, the other major phospholipid in the

Table 1. *Effect of bovine heart mitochondrial phospholipids on the reconstitution of Complex-III liposomal vesicles exhibiting control of electron flow*

The respiratory control ratio is expressed as the ratio of duroquinol-cytochrome *c* reductase activity in the presence and absence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP). Mitochondrial phospholipid (MPL) is the mixture of phospholipids in bovine heart mitochondria. It contains diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) to the extent of 20, 3, 36 and 40% of the total phospholipid phosphorus respectively (Fleischer *et al.*, 1967). The values are means $\pm$ S.E.M. for five to ten determinations.

Phospholipid	Duroquinol-cytochrome <i>c</i> reductase (μ mol of cytochrome <i>c</i> reduced/ min per mg of protein)		Respiratory control ratio
	-FCCP	+FCCP	
MPL	3.3	18.4	5.6
PE	3.2 $\pm$ 0.1	12.7 $\pm$ 0.9	4.1 $\pm$ 0.4
PC	13.1 $\pm$ 2.0	7.3 $\pm$ 1.1	0.6 $\pm$ 0.3
DPG	8.2 $\pm$ 0.4	6.8 $\pm$ 0.4	0.8 $\pm$ 0.1
PE/PC (2:1)	3.1 $\pm$ 0.3	17.9 $\pm$ 1.4	5.9 $\pm$ 0.5
(1:1)	2.2 $\pm$ 0.2	13.9 $\pm$ 1.4	6.4 $\pm$ 0.2
(1:3)	3.3 $\pm$ 0.3	16.5 $\pm$ 0.6	5.2 $\pm$ 0.4
PE/DPG (3:1)	4.4	9.1	2.1
(1:1)	6.4	10.3	1.7
PC/DPG (3:1)	8.8	9.1	1.0
(1:1)	6.6	9.8	1.5

Table 2. *Effect of soya-bean phospholipids on the reconstitution of Complex-III liposomal vesicles exhibiting control of electron transport*

For details and abbreviations see the legend to Table 1. In addition, phosphatidic acid is abbreviated as PA.

Phospholipid	Duroquinol-cytochrome <i>c</i> reductase (μ mol of cytochrome <i>c</i> reduced/ min per mg of protein)		Respiratory control ratio
	-FCCP	+FCCP	
PE	2.4 $\pm$ 0.4	12.7 $\pm$ 1.5	5.4 $\pm$ 0.5
PC	20.5 $\pm$ 2.2	20.5 $\pm$ 1.0	1.0
PI	3.0	3.0	1.0
PA	2.5	2.5	1.0
PE/PI (4:1)	11.0	24.0	2.2
(1:1)	11.0	20.0	1.8
(1:4)	9.5	10.5	1.1
PE/PA (4:1)	6.0	36.0	6.0
(1:1)	8.5	23.0	2.7
(1:4)	6.0	12.0	2.0

mitochondrial inner membrane (Table 1). Vesicles formed with phosphatidylcholine were relatively impermeable to univalent cations, as indicated by their ability to form a stable membrane potential from K^+ diffusion gradients (Wielburski & Nelson, 1978). Thus the high electron-transfer rates obtained with phosphatidylcholine vesicles in the absence of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone suggests that Complex III is not properly inserted in the membrane. Diphosphatidylglycerol, phosphatidylinositol or phosphatidic acid did not, individually, form vesicles exhibiting respiratory control (Tables 1 and 2). Thus phosphatidylethanolamine is the only major mitochondrial phospholipid that when added by itself is sufficient to reconstitute Complex III functionally with respect to respiratory control.

Respiratory control can be reconstituted with pure phosphatidylethanolamine from several sources with approximately the same efficiency (Table 3), even though the fatty-acid compositions of *E. coli* (Shukla & Turner, 1980), bovine heart (Fleischer & Rouser, 1965; Kagawa *et al.*, 1973) and soya-bean (Kagawa *et al.*, 1973) phosphatidylethanolamine differ widely. However, (dimyristoyl)phosphatidylethanolamine, under the conditions used, does not form vesicles capable of respiratory control (Table 3). The lack of respiratory control with (dimyristoyl)phosphatidylethanolamine was reported in reconstitution studies with cytochrome oxidase (Eytan *et al.*, 1977). This inability is undoubtedly related to the fact that this lipid, which has a melting transition at 48–49°C in aqueous medium (Mabrey & Sturtevant, 1976; Van Dijck *et al.*, 1976) is in the gel rather than the liquid-crystalline state.

Reconstitution of respiratory control with mixtures of phospholipids

Various combinations of bovine heart mitochondrial phospholipids were also tested in the reconstitution of Complex III. Respiratory control can be achieved with mixtures of phosphatidylethanolamine and phosphatidylcholine, even when phos-

phatidylethanolamine comprises only a fraction of the total phospholipid (Table 1). In some experiments, ratios of phosphatidylethanolamine/phosphatidylcholine as low as 1:6 (w/w) gave respiratory control values of 4.

Surprisingly, diphosphatidylglycerol destroyed respiratory control when admixed with phosphatidylethanolamine (Table 1), and did not improve respiratory control when admixed with phosphatidylcholine (Table 1). These results are in contrast with those in which diphosphatidylglycerol was shown to improve respiratory control with cytochrome oxidase (Eytan *et al.*, 1976) or oxidative phosphorylation driven by Complex I (Ragan & Racker, 1973). Soya-bean phosphatidylinositol and phosphatidic acid both supported respiratory control when mixed at low ratios with phosphatidylethanolamine (Table 2). Increasing the relative amount of phosphatidylinositol or phosphatidic acid led, however, to a decrease in respiratory control.

An optimal lipid/protein ratio of 2–3 is found with a 1:1 mixture of phosphatidylethanolamine and phosphatidylcholine (Fig. 1), in agreement with previous results (Leung & Hinkle, 1975).

Effects of phospholipids on electron-transfer rates

Enzyme activity tended to be slightly higher in vesicles containing pure phosphatidylcholine than in those containing phosphatidylethanolamine (Table 1, Fig. 1). As a result, respiratory control in mixed phosphatidylethanolamine/phosphatidylcholine vesicles is due partially to the formation of non-leaky vesicles, and partially to activation of the enzyme (Fig. 1). Diphosphatidylglycerol, phosphatidylinositol and phosphatidic acid all inhibited duroquinol–cytochrome *c* reductase activity when mixed individually with the enzyme. As indicated above, diphosphatidylglycerol also inhibited enzyme activity even when mixed with phosphatidylethanolamine or phosphatidylcholine (Table 1). In contrast, both phosphatidylinositol and phosphatidic acid stimulated phosphatidylethanolamine-supported enzyme activity when admixed at low ratios with

Table 3. *Effect of phosphatidylethanolamine from various sources on the reconstitution of Complex-III vesicles exhibiting control of electron transport*

For details and abbreviations, see the legend to Table 1.

	Duroquinol–cytochrome <i>c</i> reductase (μ mol of cytochrome <i>c</i> reduced/ min per mg of protein)		Respiratory control ratio
	–FCCP	+FCCP	
Phosphatidylethanolamine			
Bovine heart mitochondrial	3.3 $\pm$ 0.8	11.5 $\pm$ 0.6	3.7 $\pm$ 0.9
Soya bean	4.4 $\pm$ 0.7	13.3 $\pm$ 0.8	3.1 $\pm$ 0.4
<i>E. coli</i>	4.5 $\pm$ 0.5	16.6 $\pm$ 0.2	3.9 $\pm$ 0.7
Dimyristoyl	12.8	8.6	0.7

Fig. 1. Control of electron flow of Complex III as a function of the phospholipid/protein ratio

A mixture of mitochondrial phosphatidylethanolamine and phosphatidylcholine in a molar ratio of 1:1 was used for the reconstitution. Duroquinol-cytochrome *c* reductase was assayed in the presence (●) and absence (○) of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

phosphatidylethanolamine (Table 2). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone had a slight inhibitory effect on duroquinol-cytochrome *c* reductase in reconstitutions carried out with mitochondrial phosphatidylcholine and diphosphatidylglycerol (Table 1). The explanation for this observation is not clear.

Freeze-fracture studies

Freeze-fracture studies of Complex-III vesicles are shown in Plate 1. Mitochondrial phosphatidylethanolamine and phosphatidylcholine (1:1) were used in this reconstitution and vesicles with respiratory control ratio of 5–6 were obtained. The reconstituted preparation appears as membranous vesicles varying in size from 25 to 100 nm. Particles of approx. 13.5 nm in diameter can be seen in the fracture faces. More particles were observed in the

outer (concave) than in the inner (convex) fracture faces of the vesicles.

Discussion

Bovine heart Complex III exhibits respiratory control when incorporated into phospholipid vesicles of purified phosphatidylethanolamine. Phosphatidylethanolamine is the only major phospholipid of the mitochondrial inner membrane that confers this property to Complex III. Phosphatidylethanolamine from bovine heart, soya bean or *E. coli* works equally well. Therefore the specificity of phosphatidylethanolamine is referable to the polar head group rather than the fatty acid moiety; the latter differs appreciably in the various phosphatidylethanolamine preparations that were used (Fleischer & Rouser, 1965; Kagawa *et al.*, 1973; Shukla & Turner, 1980). The specific requirement for phosphatidylethanolamine is readily observed when admixed with phosphatidylcholine in the reconstitution. Phosphatidylcholine by itself does not support respiratory control in Complex III. However, when phosphatidylethanolamine is admixed with phosphatidylcholine as only a small fraction of the phospholipid, respiratory control is restored. Since vesicles formed on reconstitution of Complex III with phosphatidylcholine are impermeable to K^+ (Wielburski & Nelson, 1978) or protons, low respiratory control observed must indicate that Complex III cannot be inserted correctly into the phosphatidylcholine bilayer. It would seem that small amounts of phosphatidylethanolamine are required for proper insertion of Complex III into phosphatidylcholine vesicles.

It has been reported that optimal respiratory control of cytochrome oxidase vesicles is achieved by including negatively charged lipids (Eytan *et al.*, 1976; Carroll & Racker, 1977). This is not the case with Complex III. Diphosphatidylglycerol inhibits both enzyme activity and respiratory control of Complex III, even when admixed with other phospholipids. Furthermore, although phosphatidylinositol and phosphatidic acid activate electron transport in mixed lipid vesicles, respiratory control is not improved over that obtained with vesicles of pure phosphatidylethanolamine.

The requirement of phospholipid for mitochondrial electron transport, including Complex III, was demonstrated many years ago, but the type of phospholipid required was non-specific (Fleischer *et al.*, 1962; Brierley *et al.*, 1962). It is intriguing that there now seems to be specificity for phosphatidylethanolamine for respiratory control of Complex III. This specificity appears to be related to the proper insertion of Complex III into the bilayer. It should be stressed, however, that the lipid specificity observed for the isolated enzyme might not reflect

EXPLANATION OF PLATE 1

Freeze-fracture electron microscopy of reconstituted Complex III

Mitochondrial phosphatidylethanolamine and phosphatidylcholine were used in a molar ratio of 1:1. The respiratory control ratio was 5–6. Two distributions of sealed vesicles can be observed, a smaller size (25–40 nm) and larger size (80–100 nm). The particles of approx. 13.5 nm, indicated by the small arrows, are referable to Complex III. Most of the particles were observed in the outer (convex) fracture face. The direction of shadowing is indicated by the large arrow.

that of the intact membrane. In the latter case protein-protein interactions could provide specificity for insertion.

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