An active cytochrome *c* oxidase that has no tightly bound cardiolipin

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Bovine and dogfish (*Squalus acanthias*) heart submitochondrial particles and cytochrome *c* oxidase (EC 1.9.3.1) were prepared. Biphasic Eadie–Hofstee plots from steady-state polarographic assays were obtained for both species. Phospholipid analyses indicated that cardiolipin was absent from this active dogfish enzyme.

It is well established that, at low ionic strength, polarographic steady-state kinetic assays of cytochrome *c* oxidase (EC 1.9.3.1) yield biphasic Eadie–Hofstee plots (Ferguson-Miller *et al.*, 1976). This behaviour has been interpreted in terms of the presence of both high- and low-affinity sites for the substrate, cytochrome *c*. It is also known that bovine oxidase contains 2–3 molecules of tightly bound cardiolipin/haem *aa*₃ (Robinson & Capaldi, 1977; Robinson *et al.*, 1980) and that these cannot be replaced by detergent without loss of activity (Vik *et al.*, 1981; Robinson, 1982). It has been suggested that this cardiolipin is essential for the activity of bovine oxidase. In addition, this phospholipid has also been implicated in the binding of cytochrome *c* to the enzyme (Bisson *et al.*, 1980; Vik *et al.*, 1981). Thus the biphasic Eadie–Hofstee plots may reflect the presence of cardiolipin at one (at least) of the substrate-binding sites.

In the present paper we report evidence that dogfish (*Squalus acanthias*) cytochrome *c* oxidase, assayed either in submitochondrial particles or isolated in detergent, exhibits biphasic steady-state kinetics. However, lipid analysis shows that the mitochondrion of this organism contains small amounts of cardiolipin and that none of this phospholipid is associated with cytochrome *c* oxidase when isolated. The hypothesis that cardiolipin plays a very specific role in the binding of cytochrome *c* and thus in the activity of oxidase is not therefore generally applicable to this enzyme.

Materials and methods

Materials

Submitochondrial particles and cytochrome *c* oxidase were isolated by the method of Yonetani (1960) from bovine heart and dogfish hearts. The purified enzyme was divided into two portions; one was dissolved in 0.1M-sodium phosphate buffer,

pH 7.4, containing 1% Tween 80; the other in 0.1M-sodium phosphate buffer, pH 7.4, containing 1mM-Triton X-100. Cytochrome *c* (type III), Sepharose 4B, Tween 80, cacodylic acid, Triton X-100 and standard phospholipids and fatty acids were purchased from Sigma Chemical Company, silica gel G-60 was from Merck, and silicic acid Bio-Sil A was from Bio-Rad Laboratories. All other chemicals were analytical-reagent grade.

Methods

Phospholipid analysis. Lipids were extracted from the samples in Triton X-100 by the method of Bligh & Dyer (1959). The extract [150–200mg of total lipid in 2 ml of chloroform/methanol (1:1, v/v)] was separated on an activated silicic acid column (22 cm × 1 cm; Bio-Sil A; 200–400 mesh) eluted with methanol/chloroform mixtures in which the percentage of methanol was increased in steps. Phospholipid fractions were further separated by using two-dimensional t.l.c. on silica-gel G-60 plates (20 cm × 20 cm) with chloroform/methanol/NH₃ (260:19:9, by vol.) and chloroform/methanol/acetic acid/water (450:75:75:18, by vol.) as solvents I and II respectively. Spots were located with I₂ vapour and identified by comparison with authentic standards.

Phospholipids were quantified by g.l.c., after conversion into their methyl esters (Metcalf & Schmitz, 1961), by using a glass column [6 ft × 0.25 in (1.84 m × 6.35 mm)] packed with 15% diethyleneglycol succinate on Gaschrom Q (100–120 mesh) at 185°C.

Cytochrome *c* oxidase activity measurements. The activity was assayed polarographically using an oxygen electrode (Rank Brothers, Cambridge, U.K.), with ascorbate/tetramethylphenylenediamine/cytochrome *c* as substrate. The method of Ferguson-Miller *et al.* (1976) was used, except that the pH was 6.5, to decrease the background O₂

Fig. 1. *Eadie-Hofstee plots of cytochrome c oxidase activity using cytochrome c as substrate*
 (a) Submitochondrial particles. Symbols: ○, bovine 10 nM-cytochrome oxidase; □, dogfish 3.4 nM-cytochrome oxidase. (b) Isolated cytochrome c oxidase. Symbols: ●, bovine 70 nM-cytochrome oxidase; ■, dogfish 25 nM-cytochrome oxidase. The assay mixture contained 25 mM-cacodylate/Tris buffer, pH 6.5, 250 mM-sucrose, 6.6 mM-sodium ascorbate and 0.66 mM-tetramethylphenylenediamine in a final volume of 3 ml. Oxidase concentrations are expressed in terms of functional units (i.e. a two-haem *a* unit).

Table 1. *Steady-state kinetic parameters and cardiolipin content of submitochondrial particles and cytochrome c oxidases*
 Catalytic-centre activity is expressed in terms of molecules of cytochrome *c* oxidized/s per oxidase functional unit.

		Bovine heart			Dogfish heart		
Site		K_m (M)	Catalytic-centre activity (s ⁻¹)	Cardiolipin (% of total phospholipids)	K_m (M)	Catalytic-centre activity (s ⁻¹)	Cardiolipin (% of total phospholipids)
Sub- mitochondrial particles	High affinity	13.5×10^{-8}	54.9	17.9	19.0×10^{-8}	149.2	3.0
	Low affinity	1.43×10^{-6}	104.4		2.5×10^{-6}	254.0	
Isolated oxidase	High affinity	12.5×10^{-8}	5.7	16.1 (approx. 4–5 mol of cardiolipin/ mol of haem <i>a</i>)	14.2×10^{-8}	12.7	0
	Low affinity	1.0×10^{-6}	8.6		1.43×10^{-6}	23.2	

consumption by ascorbate. Cytochrome *c* concentration was increased from 0.36 μ M to 14.15 μ M in the reaction mixture.

Results and discussion

Figs. 1(a) and 1(b) show the results of polarographic steady-state kinetics assays of cytochrome *c* oxidase either in submitochondrial particles or isolated in purified form. For both species studied, ox and dogfish, the Eadie-Hofstee plots were found to be biphasic. Taking this biphasicity to reflect the

presence of high- and low-affinity sites (Ferguson-Miller *et al.*, 1976, 1978), we have calculated the K_m values and the catalytic-centre activities for these sites (see Table 1). Our results for bovine oxidase are in general agreement with those reported by Ferguson-Miller *et al.* (1976). In particular, we note K_m values of approx. 100 nM for the higher- and approx. 1 μ M for the lower-affinity site. Also, the purified enzyme is distinctly less active than when incorporated into submitochondrial particles, as expected from the known lipid activation of the enzyme.

The results with the dogfish enzyme are very similar to those determined for the bovine enzyme, both generally and in the K_m values (see Table 1). The activity of the dogfish enzymes, as previously noted (Wilson *et al.*, 1980), is higher under these conditions than that of the bovine enzyme. These general similarities in the steady-state kinetic properties of cytochrome *c* oxidases from diverse organisms are in agreement with previous reports (Darley-Usmar *et al.*, 1981).

Table 1 also shows some results of phospholipid analysis of submitochondrial particles and the isolated enzyme. In agreement with Vik *et al.* (1981) and Robinson *et al.* (1980), we have found that bovine mitochondria contain cardiolipin, and that approx. 4–5 molecules of this lipid/haem *a* is associated with the isolated enzyme. In addition, we have found, in agreement with Robinson & Capaldi (1977), that passage of this isolated bovine enzyme through a gel-filtration column (Sephadex 4B equilibrated with Triton X-100) largely removes other phospholipids, but still leaves approx. 3 molecules of cardiolipin per haem *a* tightly bound to the enzyme. The lipid analysis of the dogfish oxidase stands in contrast with the results given above. Very little cardiolipin is present in dogfish mitochondria and none at all (<0.1 mol of cardiolipin/mol of haem *a*) is associated with the isolated enzyme. Passage through a Sephadex 4B column equilibrated with buffer containing Triton X-100 removed all phospholipids from the isolated dogfish enzyme and results in complete loss of activity.

As the dogfish enzyme as isolated is active, yet contains no cardiolipin, we interpret our results to mean that cardiolipin is not universally required by oxidase for activity and that in the dogfish enzyme it cannot therefore constitute part of the cytochrome *c*-binding site. In this context it is interesting to note two previous reports of oxidases (from yeast and *Nitrobacter agilis*), which also have no essential

requirement for cardiolipin for activity (Watts *et al.*, 1978; Fukumori & Yamanaka, 1982).

It is possible that other lipids substitute for the roles played by cardiolipin in the bovine enzyme. When compared with the bovine enzyme, elevated amounts of another negatively charged phospholipid, phosphatidylinositol, were detected in the dogfish oxidase, suggesting that this might have replaced cardiolipin.

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