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Functional Binding of Cardiolipin to Cytochrome *c* Oxidase

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Bovine cytochrome *c* oxidase usually contains 3–4 mol of tightly bound cardiolipin per cytochrome aa_3 complex. At least two of these cardiolipins are required for full electron transport activity. Without the tightly bound cardiolipin, cytochrome *c* oxidase has only 40–50% of its original activity when assayed in detergents that support activity, e.g., dodecyl maltoside. By measuring the restoration of electron transport activity, functional binding constants for cardiolipin and a number of cardiolipin analogues have been evaluated ($K_{d,app} = 1\,\mu M$ for cardiolipin). These binding constants agree reasonably well with direct measurement of the binding using [^{14}C]-acetyl-cardiolipin ($K_d < 0.1\,\mu M$) when the enzyme is solubilized with Triton X-100. These data are discussed in relationship to the wealth of data that is known about the association of cardiolipin with cytochrome *c* oxidase and the other mitochrondrial electron transport complexes and transporters.

KEY WORDS: Cytochrome *c* oxidase; cardiolipin; diphosphatidylglycerol; electron transport activity; binding; synthesis of cardiolipin analogues; spin-labeled cardiolipin; azido-cardiolipin; photolabeling.

INTRODUCTION

Cardiolipin (diphosphatidylglycerol) is a unique phospholipid found only in membranes that function in electron transport, i.e., mitochrondrial inner membranes, chloroplast thylakoid membranes, and bacterial plasma membranes. In eukaryotes, cardiolipin (CL) is synthesized exclusively within the mitochrondrion and is found predominantly in the mitochrondrial inner membrane (Ioannou and Golding, 1979). The structure of CL (Fig. 1) is quite different from all other phospholipids since it contains three glycerols, two phosphates, and four unsaturated fatty acyl tails (approximately 80% of them are linolenyl groups). Unlike the other major phospholipids of the inner mitochrondrial membrane, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), CL is acidic and negatively charged. Because of the unique cardiolipin content of these membranes, experiments for almost 30 years have probed its possible participation, or functional involvement, in coupled electron transport.

It is now known that cardiolipin is probably not a passive component of the inner mitochrondrial membrane, but it interacts with a number of the inner mitochondrial membrane proteins. Nearly all of the electron transport complexes have been reported to require cardiolipin for maximum electron transport activity, e.g., Complex I (Fry and Green, 1981; Goormaghtigh *et al.*, 1986), Complex III (Nicolay and deKruijff, 1987), cytochrome *c* oxidase (the subject of this review), and glycerol-3-phosphate dehydrogenase (Beleznai and Jancsik (1989)). Each can be isolated as a detergent-solubilized, multisubunit, protein–lipid complex that contains 10–60 mol of phospholipid. When assayed in the proper detergent environment, e.g., dodecyl maltoside, Tween 20, or Tween 80, each has electron transport activity that is similar to the activity it had in the mitochrondrial membrane. However, if the endogenous phospholipid is completely removed, or sequestered by CL binding drugs, electron transport activity usually decreases. With each complex, removal of CL has been suggested as a possible cause of decreased activity, and nearly full

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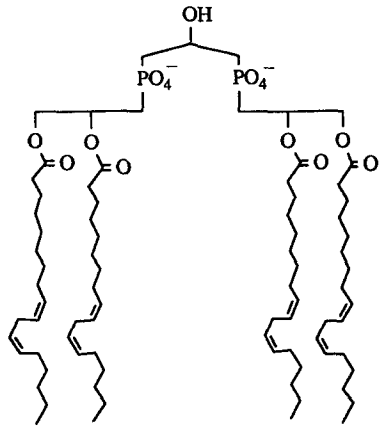

Fig. 1. The structure of cardiolipin (diphosphatidylglycerol).

activity can usually be restored by exogenous CL, but not by other PL's. In addition to these effects, CL has also been proposed to directly participate in proton pumping and may be one of the predominant causes of proton conductance, i.e., proton leak, during state 4 respiration (Hoch, 1992).

Activity of several of the mitochrondrial translocases has also been shown to be dependent upon the presence of CL; however, with the exception of the ADP/ATP translocase, the evidence for specific CL binding is quite weak. The only evidence that CL binds to either the monocarboxylate carrier or carnitine/acylcarnitine translocase is the increased stability and slightly higher activity of these proteins if they are isolated in the presence of CL (Nalecz *et al.*, 1986; Noel and Pande, 1986). With the phosphate carrier the evidence is somewhat better since it is known to be stabilized by CL, has twofold higher activity in detergent or PL mixtures containing CL (Kadenbach *et al.*, 1982; Kaplan *et al.*, 1986; Mende *et al.*, 1982, 1983), and is inhibited by an analogue of adriamycin (Cheneval *et al.*, 1983). Only the ADP/ATP carrier has been shown to definitely contain tightly bound CL (Kramer and Klingenberg, 1980; Brandolin *et al.*, 1980; Beyer and Klingenberg, 1985), but the functional importance, specificity, or affinity of CL has not been measured. Cardiolipin may also serve another unrelated function, targeting of cytoplasmically synthesized preproteins to the mitochrondria. Specific binding of leader presequences to anionic phospholipids, e.g., CL, has been proposed to be the first step in mitochrondrial import of these proteins (Ou *et al.*, 1988; Myers *et al.*, 1987; Tamm, 1986; Cheneval and Carafoli, 1988; Muller *et al.*, 1985; Demel *et al.*, 1989; Eilers *et al.*, 1989). Since CL is unique to mitochrondria, affinity of presequences for CL could be a selective mechanism by which these proteins are directed into this subcellular organelle. Thus, specific association of CL with proteins and preproteins within the inner mitochrondrial membrane appears to be a general, rather than a unique situation.

However, most of the evidence for the association of CL with specific mitochrondrial proteins or preproteins is fairly speculative. Direct binding of CL to specific proteins is only inferred from indirect functional studies, i.e., (1) the influence of exogenous CL upon electron transport, or translocase activities; or (2) the effect of adding compounds known to sequester CL within phospholipid bilayers, e.g., adriamycin, upon these rates. Because these approaches are usually limited to a positive or negative result, little is known about the details of the CL interaction with each protein. In general almost nothing is known about structural specificity, binding affinity, and existence of specific binding sites, whether the interactions are predominantly polar, ionic, or hydrophobic, or if, in fact, the effects are merely the result of a general stimulation in activity by acidic phospholipids. The single exception is cytochrome *c* oxidase. In this case, a large number of studies indicate a specific and tight association between cytochrome *c* oxidase and CL that is functionally important for maximal activity of at least the bovine heart enzyme. The focus of this article is to review the experimental evidence that supports the conclusion that a small number of cardiolipins (probably 2 mol/mol oxidase) specifically bind to high-affinity binding sites on bovine heart cytochrome *c* oxidase and increase the rate of internal electron transfer within this electron transport complex.

FIRST STUDIES INDICATING A FUNCTIONAL ROLE FOR CARDIOLIPIN IN CYTOCHROME *c* OXIDASE

Evidence Based Upon Depletion of Bound Cardiolipin

Although most of the phospholipids can be removed from cytochrome *c* oxidase without affecting electron transport activity, there is a subclass of phospholipids essential for its function. It has been known for many years that most "phospholipid-depleted" preparations of cytochrome *c* oxidase contain 3–4 mol CL per mol cytochrome aa_3 that are difficult to extract with either organic solvents (Brierley and Merola,

1962), or exchange with nondenaturing detergents (Awasthi *et al.*, 1971; Robinson and Capaldi, 1977). Yu *et al.* (1975) were the first to report the removal of most of the tightly bound CL to prepare "lipid-depleted" cytochrome *c* oxidase (ca. 1 CL/oxidase); the activity of the phospholipid-depleted oxidase was dependent upon the addition of exogenous CL, at least when the enzyme was dissolved in Emasol 1130 (a detergent similar to Tween 20). In the early 1980's, three independent studies confirmed that at least some of the tightly bound CL was required for maximum electron transport activity of bovine heart enzyme (Fry and Green, 1980; Robinson *et al.*, 1980; Fry and Green, 1981; Vik *et al.*, 1981). In each case, removal of the tightly bound CL resulted in decreased activity which could be restored only by the addition of exogenous CL, i.e., addition of other phospholipids was ineffective. The high affinity and specificity of CL binding to cytochrome *c* oxidase was further indicated by the fact that in two of these studies, recovery of full activity was possible with as little as 1.7 μM CL in the final assay mixture which contained 5–10 mM Tween 20 or Tween 80 (Robinson *et al.*, 1980; Vik *et al.*, 1981). As will be discussed in a later section on the measurement of cardiolipin binding affinity and stoichiometry, this type of functional binding assay has been used to measure apparent binding affinities of a whole series of phospholids and cardiolipin analogues (Robinson, 1982; Dale and Robinson, 1988a; Robinson *et al.*, 1990).

Evidence Based upon Preferential Interaction of Spin-Labeled Cardiolipin with Cytochrome c *Oxidase*

Concurrent with the CL delipidation studies, Cable and Powell (1980) found that cytochrome *c* oxidase exhibited some specificity for cardiolipin based upon the preferential segregation of spin-labeled cardiolipin into the boundary layer phospholipids. However, the specificity for cardiolipin did not appear to be as strong, or as selective as was implicated by the functional studies, nor was there any indication of tight binding to a small number of sites. Spin-labeled cardiolipin interacted with a large number of sites (about 40) with a partition coefficient only two times larger than phosphatidylcholine. Powell and colleagues greatly extended the spin-label technique to measure the binding of a number of different spin-labeled phospholipids and CL analogues which will be discussed in the section on measurement of cardiolipin binding and stoichiometry. As will be seen in this later section, the results obtained by the spin-label technique are qualitatively, but not quantitatively, in agreement with functional and/or direct binding experiments.

Evidence Based upon Inactivation of Cytochrome c *Oxidase by the Cardiolipin Binding Drug Adriamycin*

Supporting evidence that CL is essential for maximal cytochrome *c* oxidase activity was also obtained at this time by complementary studies using the cardiotoxic antitumor drug adriamycin. Binding of adriamycin to the head group of CL is known to be quite tight (K_d = 1 μM) with a stoichiometry of 2 adriamycin per CL (Goormaghtigh *et al.*, 1980; Goormaghtigh and Ruysschaert, 1984; Nicolay *et al.*, 1984; Cheneval *et al.*, 1985). A consequence of this interaction is the sequestering of CL from the bulk phase membrane environment into a separate lipid domain. The addition of adriamycin to mitochrondrial membranes should, therefore, prevent the association of CL with other membrane components. Indeed, adriamycin does inhibit cytochrome *c* oxidase within mitochrondrial membranes (Goormaghtigh *et al.*, 1982). Similar adriamycin inhibition of the other electron transport complexes has also been interpreted in terms of CL sequestering by adriamycin (Goormaghtigh and Ruysschaert, 1984; Goormaghtigh *et al.*, 1982; Goormaghtigh *et al.*, 1986). Since this inhibition of electron transport activity by adriamycin occurs either in membranes, or with detergent-solubilized complexes, these experiments appear to indicate that conclusions based upon CL depletion experiments are not artifacts of solubilization and delipidation procedures, but reflect the true importance of CL associations within the membrane.

Inhibition of cytochrome *c* oxidase and other electron transport complexes by adriamycin, however, may not be a direct CL effect. Adriamycin is known to form complexes with trace amounts of metal ions, e.g., ferric ion, and these complexes cause peroxidation of polyunsaturated fatty acyl groups (Demant, 1983; Demant and Jensen, 1983). In addition, the ferric-adriamycin complex itself competitively and reversibly inhibits cytochrome *c* oxidase (Hasinoff and Davey, 1988). In fact, Demant has recently concluded that, at least in submitochrondrial particles, inactivation of cytochrome *c* oxidase by adriamycin does not correlate with its binding to membrane lipids, i.e., CL, but rather with the generation of oxygen free radicals and the iron-dependent peroxidation

reactions. Clearly, the mechanism of adriamycin inactivation of cytochrome *c* oxidase and the other electron transfer components is complex. Any conclusion that CL is directly involved in the activity of any of the inner membrane electron transfer complexes based upon inhibition by adriamycin should probably not be used as supportive evidence unless extreme care is taken to avoid the complications introduced by these free radical and peroxidation reactions.

METHODS FOR REMOVING ENDOGENOUS CARDIOLIPIN FROM CYTOCHROME *c* OXIDASE AND QUANTITATION OF BOUND CARDIOLIPIN

Two basic approaches are available for the extraction of the tightly bound CL from cytochrome *c* oxidase; detergent exchange (Yu *et al.*, 1975; Robinson *et al.*, 1980, 1990; Robinson, 1982) and phospholipid exchange (Watts *et al.*, 1978; Powell *et al.*, 1985; Adramovitch *et al.*, 1990). In the first method, an excess of nondenaturing detergent, usually cholate or Triton X-100, is added and the resulting detergent–protein complex is separated from phospholipid-detergent mixed micelles by either glycerol gradient centrifugation or ammonium sulfate precipitation. I have preferred the centrifugation method since it avoids the successive precipitation steps that often cause partial loss of activity. Both higher ionic strength and higher pH favor removal of CL by detergents, but such conditions also increase the probability of subunit removal, particularly subunits III, VIa, and VIb (Robinson and Wiginton, 1985). The second approach is to repeatedly exchange CL with synthetic phospholipids, usually dimyristoylphosphatidylcholine (DMPC), by ammonium sulfate precipitation in the presence of cholate (Powell *et al.*, 1985). This method effectively exchanges DMPC for CL, but does not generate a soluble detergent–protein complex and necessitates the measurement of residual CL by methods other than determination of lipid phosphorus as discussed below. We have recently begun to use a third method for the removal of CL: incubation of oxidase with a mixture of 5% (w/v) Triton X-100 and 0.5% (w/v) myristyltrimethylammonium chloride followed by isolation of the resulting delipidated complex by ion exchange chromatography on DEAE Sephacel. Addition of the cationic detergent facilitates extraction of CL, probably due to its more favorable partition into the mixed micelles, and ion exchange chromatography is certainly a much easier method to use with large amounts of protein than glycerol gradient centrifugation.

Regardless of the procedure used for removal of CL, the major problem is an accurate determination of residual CL after its extraction. An accuracy of at least ± 0.2 CL per cytochrome aa_3 is required for any type of functional correlation study. The usual approach is to measure residual lipid phosphorus; however, this is complicated by three factors: (1) Even if 1 mg of oxidase is analyzed for residual CL, CL must be measured with an accuracy of at least ± 1 nmol which is fairly close to the limits of accuracy of the normal chemical assays for phosphorus; (2) Residual CL is very difficult to extract with most organic solvents; e.g., acetone–water (90 : 10, v/v) or chloroform–methanol (2 : 1, v/v) extract only 50–60% of the bound CL (Awasthi *et al.*, 1971) with a similar, but less severe, incomplete extraction when CL is extracted according to Bligh and Dyer (1959) at neutral pH (unpublished results); and (3) lipid phosphorus due to residual CL must be accurately measured either in the presence of high concentrations of detergents, which usually contain phosphorus-containing contaminants, or relatively high concentrations of other exogenous phospholipids. Wet ashing of at least 1 mg of the entire protein–phospholipid–detergent complex certainly avoids problems 1 and 2, but probably overestimates the amount of bound CL due to phosphorus-containing contaminants. We have used three approaches to avoid this problem (Robinson *et al.*, 1990): (1) Determine total phosphorus in lipids extracted according to Bligh and Dyer after partial denaturation of the enzyme in 2 M guanidinium chloride (conditions of guanidinium denaturation are based upon Hill *et al.* (1988); (2) determination of total phosphorus after dry acetone extraction of detergents from completely dry, lyophilized complex; or (3) extraction of phospholipids according to Bligh and Dyer in the presence of 0.1 M hydrochloric acid, conditions that maximize the extraction of CL and minimize extraction of phosphorus-containing contaminants, followed by removal of Triton X-100 by thin layer chromatography with dry acetone. The first two methods usually give slightly higher values than the third method, usually 0.25–0.5 more CL/oxidase. We recently

found that non-CL, but phosphorus-containing compounds generally contaminate the CL extracted according to the second method since a significant amount of the extracted lipid phosphorus does not elute as CL when analyzed by silicic acid HPLC according to Robinson (1990) (unpublished results). Therefore, the values of 0.5–1.0 mol of residual CL per oxidase in our low-lipid preparations (e.g., Robinson *et al.*, 1990) overestimate the amount of bound CL by a factor of at least of 3.

Methods other than total lipid phosphorus must be used to measure residual CL when it is removed from oxidase by exchange with DMPC (Powell *et al.*, 1985). Because CL is the only phospholipid containing $C_{18:2}$ fatty acyl tails, and the methyl esters have little or no affinity for cytochrome *c* oxidase, Powell and coworkers have used quantitative determination of methyl linoleate esters by gas–liquid chromatography as a reliable method for determining residual CL. This approach is reliable and gives results that are quite comparable with those we have obtained based upon total lipid phosphorus.

STRUCTURAL REQUIREMENTS OF CARDIOLIPIN FOR RESTORATION OF CYTOCHROME *c* OXIDASE ACTIVITY

As described above, CL-depleted cytochrome *c* oxidase only has 30–50% of the electron transport activity of oxidase containing 3–4 tightly bound CL's when assayed in detergents that support high activity, e.g., dodecyl maltoside, Tween 20, and Tween 80. Restoration of activity is hyperbolically dependent upon the concentration of added exogenous CL or CL analogues. Therefore, the change in activity upon adding CL which is dissolved in detergent micelles has been used to evaluate the relative binding constants of a whole series of CL analogues (Robinson, 1982; Dale and Robinson, 1988b; Robinson *et al.*, 1990).

Preparation of Cardiolipin Analogs

A series of CL analogues has been synthesized to probe the structural features of CL that are essential for the recovery of full functional activity. All syntheses followed a similar approach; acylation of a free hydroxyl group within either CL, monolyso-CL (MLCL), or dilyso-CL (DLCL) with a carboxylic acid, sometimes after reversibly blocking the 2-hydroxyl on the bridge glycerol with tetrahydropyran (Dale and Robinson, 1988a). CL analogues that have been successfully prepared are listed in Table I.

Functional Binding Constants of Cardiolipin and Its Analogues in Detergent

Reassociation of the CL analogues with the CL-depleted oxidase (< 0.5 CL/cytochrome aa_3) is not always instantaneous; therefore, this type of experiment usually involves incubation of oxidase with CL, or a CL analogue often in a buffer containing Triton X-100, followed by dilution into a spectrophotometric electron transfer assay in dodecyl maltoside. Triton X-100 has been used during the incubation with the CL's since reassociation of CL with oxidase was much more rapid in Triton X-100 than in dodecyl maltoside, a few minutes rather than hundreds of minutes. Full restoration of activity to CL-depleted cytochrome *c* oxidase was found to be highly specific for CL (19,20). No other phospholipids, e.g., phosphatidylglycerol, phosphatidic acid, phosphatidylserine, phosphatidylcholine, or phosphatidylethanolamine, was able to restore activity. However, many of the CL analogues listed in Table I were nearly as effective as CL itself. By testing each of these derivatives, the structural features of CL that are essential for binding, $K_{d,app}$, and restoration of activity, DMA, have been defined (Table II) (1,6). The data in Table II are best summarized as follows: (1) CL binds to cytochrome *c* oxidase with high affinity, $K_d = 1\,\mu M$, even in the presence of high concentrations of detergent (the K_d corresponds to about 0.1 CL per micelle); (2) modifying the hydroxyl on the polar end of CL has little effect on either the affinity or the maximum restored activity, e.g., similar results were obtained for CL, AcCL, SucCL THP-CL, and O_3CL; similar results for MLCL and Ac_2MLCL; and also for DLCL and Ac_3DLCL; (3) Replacing two of the $C_{18:2}$ groups in CL either by $C_{18:1}$ (O_2CL) or $C_{14:0}$ has little affect on either the affinity or ability of CL to restore activity; removal of a single acyl chain, e.g., MLCL, also has only a minor effect; (4) Both the affinity and ability to restore activity decrease when acyl groups are replaced by short chain fatty acids (H_2CL), and binding is very poor if they are removed (DLCL) or replaced by acetyl groups (Ac_3DLCL).

Functional Simulation by Cardiolipin in Pure Phospholipid

An alternative to the above approach is to measure the increase in the activity of CL-depleted oxidase

Table I. Cardiolipin Analogues That Have Been Synthesized and Purified

Compound	Group(s) modified	Attached group	Reference
[^{14}C]-AcCL	2-Hydroxyl	[1-^{14}C]-acetic acid	*a*
Ac_2MLCL	2-OH and one 2′-OH	Acetic acid	*a*
Ac_3DLCL	2-OH and both 2′-OH's	Acetic acid	*a*
Suc-CL	2-Hydroxyl	Succinic acid	*a*
THP-CL	2-Hydroxyl	Tetrahydropyran	*b*
THP-MLCL	2-OH; (2′-OH free)	Tetrahydropyran	*c*
O_2CL	Both 2′-hydroxyls	Oleic acid	*b*
M_2CL	Both 2′-hydroxyls	Myristic acid	*b*
H_2CL	Both 2′-hydroxyls	Hexanoic acid	*b*
O_3CL	2-OH and both 2′-OH's	Oleic acid	*b*
2′-[Arylazido-C_{12}]-CL	One 2′-hydroxyl	12-[*N*-(4-azido-2-nitrophenyl)]-aminododecanoic acid	*c*

[a] Robinson *et al.* (1990).
[b] Dale and Robinson (1988a).
[c] Dale and Robinson (1988b).

by pure phospholipids. Because the added phospholipid will coat the entire surface of the protein that spans the membrane, activity measurements are a combination of two effects: how well the phospholipids can meet the general apolar requirements of cytochrome *c* oxidase, and whether they reassociate with the CL-binding sites. Such studies were first done by Yu *et al.* (1975) as well as more recently by Adramovitch *et al.* (1990). In the first case, CL was not as effective as asolectin, phosphatidylcholine, or phosphatidylethanolamine. In the second case, the order of stimulation was: acylated-CL (similar to O_3CL above) > CL > DLCL = DMPC. Adramovitch *et al.* have preferred not to refer to this stimulation in activity as the "restoration of full activity" although they observe the same twofold change in activity as described above in the detergent studies.

Table II. Restoration of Activity to Cardiolipin-Depleted Cytochrome *c* Oxidase by Derivatives of Cardiolipin

Compound	$K_{d,app}$ (μM)	$\Delta(MA)_{max}$ (sec^{-1})	Reference
CL	0.7–1.3	100	*a,b*
AcCL	0.4	90	*a*
2′-[Arylazido-C_{12}]-CL	1.0	95	*c*
Suc-CL	0.5	90	*a*
THP-CL	1.3	95	*b*
O_3CL	1.5	80	*b*
O_2CL	1.0	100	*b*
M_2CL	1.2	70	*b*
MLCL	1.7	65	*a*
Ac_2MLCL	2.2	63	*a*
H_2CL	6.8	55	*a*
DLCL	> 20	0–30	*a,b*
Ac_3DLCL	> 20	0–30	*a*
PA	> 20	40–70	*a*
PG	> 20	50–90	*a*

[a] Robinson *et al.* (1990).
[b] Dale and Robinson (1988a).
[c] Dale and Robinson (1988b).

DIRECT MEASUREMENT OF THE AFFINITY AND STOICHIOMETRY OF CARDIOLIPIN ANALOGUES FOR CARDIOLIPIN-DEPLETED CYTOCHROME *c* OXIDASE

By correlating the amount of residual tightly bound CL in cytochrome *c* oxidase with the remaining electron transport activity and also with direct and competition binding measurements of [^{14}C]-AcCL to CL-depleted cytochrome *c* oxidase, we have concluded that detergent-solubilized cytochrome *c* oxidase requires 2 mol of tightly bound CL per monomeric complex for full functional activity (Robinson *et al.*, 1990). We have also concluded that it is the reassociation of these 2 mol of CL that was responsible for restoration of activity to CL-depleted cytochrome *c* oxidase discussed above. By measuring the ability of other PL's to compete for [^{14}C]-AcCL binding, we were also able to measure the binding constants for CL, MLCL, and DLCL. These data are summarized in Table III.

Based upon these direct binding data, we have concluded the following: (1) *Specific CL binding*: CL-depleted cytochrome *c* oxidase has two high-affinity binding sites for AcCL, $K_d < 0.1\ \mu M$. These sites are not present in enzyme containing endogenous CL; (2)

Table III. Binding Constants and Stoichiometry for Cardiolipin, Monolysocardiolipin, and Dilysocardiolipin Binding to Cardiolipin-Depleted Cytochrome *c* Oxidase[a]

Phospholipid	$K_{d,1}$ (μM)	$v_{max,1}$ (PL/complex)	$K_{d,2}$ (μM)	$v_{max,2}$ (PL/complex)
AcCL	0.04	1.8	4	2.5
CL	0.07	1.8	4	2.5
MLCL	0.4	1.8	4	2.5
DLCL	4	1.8	4	2.5
PC	> 200	—	> 200	—

[a]Data are from Robinson *et al.* (1990).

Nonspecific CL binding: two to three additional lower-affinity binding sites, $K_d = 4\,\mu$M, are present in both the CL-depleted and non-delipidated complexes; (3) *Relative affinities of CL analogs*: Binding affinities of CL, MLCL, and DLCL as measured by the direct binding method (Table III) are similar to those obtained by the functional assay approach (Table II); (4) Conclusions based upon functional studies are in agreement with the direct binding data, i.e., the relative affinities of the PL's are the same by both approaches.

SPECIFICITY OF CYTOCHROME *c* OXIDASE FOR CARDIOLIPIN AS ASSESSED BY SPIN LABEL TECHNIQUES

Immobilization of spin-labeled cardiolipin by CL-depleted cytochrome *c* oxidase has also been used to probe the affinity of oxidase for CL and other phospholipids (Knowles *et al.*, 1981; Powell *et al.*, 1985, 1987; Marsh and Powell, 1988). Although a distinct preference was found for CL, no evidence for a small subclass of tight CL sites was observed. Rather the data fit CL binding to 40–50 sites, i.e., the entire boundary layer region, with an affinity that is only 4.6–5.5 times greater than PC. Some specificity for spin-labeled MLCL and acyl-CL was also found since their affinity was 3.9 and 1.4 times higher than PC respectively (Powell *et al.*, 1987).

Clearly, the spin-label experiments are not consistent with either the functional apparent binding constants in Table II, or the direct binding measurements in Table III. Although complete agreement may not be expected since the experiments are done so differently and are sensitive to different factors, it would seem that the results should be closer. Why two different approaches have produced such different results is not at all clear. However, the spin-label studies do necessitate a complex curve fitting of the rather complex EPR spectrum, something that is not required in either of the two other approaches. Since the spectrum is satisfactorily fitted by a two-state model, i.e., freely mobile and boundary-layer CL, there is no need to postulate a third class of one to two tightly bound CL's. Whether such a minor component of tightly associated CL would be visible in a background of these other two more loosely bound states is not clear since such a highly immobilized spectrum of a few molecules of very tightly bound spin-labeled CL has never been observed. However, one would expect that highly immobilized spin-labeled CL would be quite easy to detect. Thus, we are only left with unsatisfactory rationalizations of why the two sets of experiments do not agree. Several such possibilities are part of the discussion in Robinson *et al.* (1990).

CHEMICAL LABELING OF THE CARDIOLIPIN BINDING SITE WITHIN CYTOCHROME *c* OXIDASE

Several attempts have been made to identify the cardiolipin binding site within bovine cytochrome *c* oxidase. Fry *et al.* (1980) concluded that CL was associated with either subunits I, II, III, or IV based upon the presence of CL in each of the subunits after it was purified in organic solvents. Such evidence is quite tentative since CL may associate with any of the denatured protein. Chemical labeling of the CL binding site within cytochrome *c* oxidase has also been attempted using either aryl azido derivatives of CL (Dale and Robinson, 1988a; Fowler *et al.*, 1988) or benzaldehyde derivatives of CL (Kuppe *et al.*, 1987). Photolabeling of the smallest cytochrome *c* oxidase subunits, i.e., subunits VIIa, VIIb, VIIc, or VIII, was observed by Fowler *et al.* (1988) with CL containing either [^{3}H]-2-(*N*-4-azido-2-nitrophenyl)-β-alanine or [^{3}H]-12-(*N*-4-azido-2-nitrophenyl)aminododecanoic acid attached to the 2-hydroxyl group of the bridge glycerol within CL, or with the [^{3}H]-azido-aminododecanoic acid attached to the 2′-hydroxyl in place of a normally occurring linoleyl acyl group of CL (Fig. 6 in Fowler *et al.*, 1988). The [^{3}H]-azido-photolabel with 12-(*N*-4-azido-2-nitrophenyl)aminododecanoic acid singly substituted on the 2′-hydroxyl on the bridge glycerol of CL was independently synthesized by Dale and Robinson (1988a). This azido-CL was then used to photolabel CL-depleted oxidase (1 : 1 ratio of azido-CL to oxidase) and compared with similar

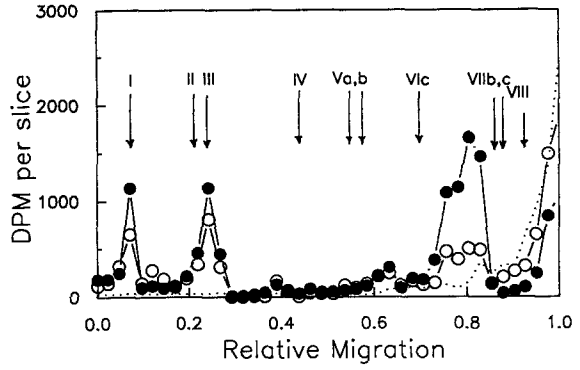

Fig. 2. Covalent labeling of CL-depleted (solid line, filled circles) and nondelipidated cytochrome *c* oxidase (solid line, open circles) by arylazido-cardiolipin. Each labeled complex was denatured in SDS, subunits separated by SDS-PAGE, and the gel sliced and counted for tritium. Arrows indicated the migration of the 13 cytochrome *c* oxidase subunit on this gel system. The dotted line is data obtained for the control where arylazido-CL was added to CL-depleted cytochrome *c* oxidase, but not irradiated (data is from Dale and Robinson, 1988b).

photolabeling of the CL containing enzyme (Fig. 2). In this case, the labeling occurred only with the CL-depleted oxidase and the labeled subunit migrated between subunits VIc and VIIa on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) prepared and run according to Kadenbach *et al.* (1983). After appropriate controls, including complete proteolysis, or phospholipase digestion of the labeled products, as well as labeling of purified subunit VIII in SDS micelles, it was concluded that: (1) the labeled subunit migrates more slowly on SDS-PAGE; and (2) the most likely site of photolabeling is subunit VIIc (subunits VIIb and VIII are less likely, but still possible, labeling sites). Experiments are presently in progress to unambiguously identify the site of azido-CL labeling.

The benzaldehyde-CL reagent has given similar but not identical results (Kuppe *et al.*, 1987). In this case, covalent labeling of non-CL-depleted cytochrome *c* oxidase by a benzaldehyde derivative of CL was compared to similar labeling by water-soluble benzaldehyde, and benzaldehyde derivatives of phosphatidylcholine and phosphatidic acid (Fig. 4 in Kuppe *et al.*, 1987). In these reagents, the benzaldehyde group was attached to the 2-hydroxyl of the bridge glycerol in CL, the phosphate group in phosphatidic acid, and the 2-hydroxyl group of lyso-PC. As we observed with azido-CL, covalent labeling of subunits with benzaldehyde-CL caused decreased migration of the labeled subunits on SDS-PAGE. In contrast to the azido-CL results, much more nonspecific labeling of subunits was obtained with each of the benzaldehyde reagents, but preferential labeling of the smaller subunits was favored, i.e., subunits V–VII.

Although the three sets of results are not entirely consistent, in total they seem to support a unique binding site that involves one or more of the smaller subunits of cytochrome *c* oxidase. One possible way to reconcile most of these results is to suggest that the apolar acyl groups of CL interact with subunit VIIb, VIIc, or VIII (consistent with the labeling pattern obtained with azido-CL) while the polar head group interacts with one of the two subunits V's or three subunit VI's (consistent with the labeling with benzaldehyde-CL).

THE FUNCTIONAL ROLE OF CARDIOLIPIN TIGHTLY BOUND TO CYTOCHROME *c* OXIDASE

A possible explanation for the functional role of the bound CL is that it is involved in the low-affinity cytochrome *c* binding site on oxidase. This was first proposed by Vik *et al.* (1981) based upon their data which showed: (1) in low ionic strength buffer containing Tween 80, oxidase depleted of CL bound only 1.2 ± 0.2 mol of cytochrome *c* per mol cytochrome aa_3 while stock enzyme bound 2.0 ± 0.3 mol of cytochrome *c* per mol of enzyme; and (2) the CL-depleted enzyme exhibited monophasic rather than biphasic cytochrome *c* kinetics. Speck *et al.* (1983) also concluded that CL may be involved in low-affinity cytochrome *c* binding based upon the altered kinetics of the CL-depleted enzyme. However, a number of kinetic studies do not support this conclusion (Thompson and Ferguson-Miller, 1983; Marsh and Powell, 1988; Adramovitch *et al.*, 1990; Ortega-Lopez and Robinson, 1993). In each of these studies, the CL-depleted enzyme still exhibits normal biphasic kinetic behavior with nearly identical values for both the high- and low-affinity K_ms, but with decreased V_{max} at both sites. This is inconsistent with the theory that CL is involved in low-affinity binding of cytochrome *c*. We have also recently shown that cytochrome *c* oxidase with and without the tightly bound CL each bind 2.0 ± 0.1 mol cytochrome *c* per mol oxidase in low ionic strength pH 7.2 buffer containing dodecyl maltoside. Both enzymes also have identical binding constants for cytochrome *c* with $K_{a1} = 8$ nM and $K_{a2} = 1$–$3\ \mu$M. We have also recently shown that the

CL-depleted enzyme has a decreased internal electron transfer rate between cytochrome *a* and the binuclear cytochrome a_3-CuB center. Therefore, bound CL is not involved in cytochrome *c* binding, but rather must influence either the conformation, or conformational flexibility of the cytochrome *c* oxidase complex, similar to the effect of varying the detergent that surrounds cytochrome *c* oxidase (Mahapatro and Robinson, 1990).

EVIDENCE AGAINST A REQUIREMENT OF CYTOCHROME *c* OXIDASE FOR BOUND CARDIOLIPIN

Although the functional, direct binding, and chemical labeling results certainly indicate a specific binding and functional role for CL tightly bound to cytochrome *c* oxidase, a number of studies appear to have reached exactly the opposite conclusion, i.e., CL is not essential for cytochrome *c* oxidase electron transport activity, or does not tightly bind to oxidase. For example, (1) cytochrome *c* oxidase isolated from yeast (*Saccharomyces cerevisae*), dogfish (*Squalus acanthias*), and rat liver all seem to have high electron transport activity and exhibited biphasic cytochrome *c* kinetics even when the isolated enzyme contains less than 1 mol CL per mol oxidase (Watts *et al.*, 1978; Al-Tai *et al.*, 1983, 1984; Thompson and Ferguson-Miller, 1983); and (2) the data from spin-label CL analogues are not consistent with highly specific binding. In addition, when the gene responsible for cardiolipin synthesis in *Escherichia coli* has been disrupted, and resulted in nondetectable cardiolipin synthase activity, aerobic growth of these strains is only somewhat slower than wild-type strains, suggesting a nonessential role for CL in aerobic metabolism, at least in *E. coli* (Nishijima *et al.*, 1988). However, low but definite levels of CL were still synthesized in these mutants due to a second gene activity, ruling out any definitive answer.

In spite of the fact that many of these results are difficult to reconcile with the previously discussed studies on bovine heart enzyme, it seems unlikely that all of the data collected on the bovine heart enzyme is invalid. Thus we are left with the difficult task of trying to rationalize why experiments done by different groups on enzymes obtained from different species and tissue types apparently disagree. A number of plausible but not convincing explanations are possible. First, the data obtained with delipidated preparations of the bovine enzyme may be incorrect, or artifactual. This does not seem reasonable since: (1) a large number of both functional and direct binding data have been obtained over a ten year span in our laboratory; (2) a very large number of different methods have been used to prepare CL-depleted oxidase with essentially the same result; (3) CL chemical labeling studies from our group seem to be in reasonable agreement with others doing this experiment, i.e., the stimulation of activity results in Adramovitch *et al.* (1990) and Marsh and Powell (1988) are really quite similar to those from my laboratory.

Second, the bovine heart enzyme may be different from cytochrome *c* oxidase isolated from other tissues and/or species, i.e., the CL effect may be bovine heart specific. Although this explanation does not seem to be entirely satisfactory, the subunit composition of oxidase isolated from each of these sources is known to be different, particularly in the smaller subunits.

Third, as mentioned earlier, quantitation of residual CL is very difficult and subject to large experimental errors. In many cases, the methods used to quantitate residual CL are not described in detail and it is difficult to determine the accuracy of the CL determinations.

Lastly, at least the functional studies are not in as much disagreement as appears in the discussions and abstracts of each study. For example, none of our functional studies have ever indicated an absolute requirement for CL for electron transport activity. Rather, we have concluded that removal of CL decreases electron transport 60–70% of its original activity and recovery of full activity is dependent upon the addition of exogenous CL. Thus, the conclusion of Watts *et al.* that "there is no absolute requirement of cardiolipin for oxidase activity" is not really in disagreement with our studies since both groups find increased activity in the presence of CL. The difference in interpretation then is based upon whether this is a restoration of intrinsic activity, or the simulation in activity, favored by Watts, Marsh, and Powell. I obviously favor the theory that reassociation of CL restores the original intrinsic activity since our data on the reassociation of CL with the CL-depleted oxidase indicates that its electron transport activity is quite similar to that of the original nondelipidated enzyme.

CONCLUSION

The functional role of CL in cytochrome *c* oxidase is still not completely understood, nor is the tight association of CL with oxidase universally accepted. However, a great deal of experimental evidence indicates that a few molecules of CL do specifically bind to oxidase and influence the rate of electron transport. The mechanism by which CL affects the catalytic transfer of electrons between cytochrome *c* and oxygen is not clear, but it appears that CL is not involved in the binding of cytochrome *c* to oxidase at either the low- or high-affinity binding sites. Rather, most of the kinetic data, and recent data from our laboratory, indicate that CL influences the rate of internal electron transfer between cytochrome *a* and the binuclear cytochrome a_3-Cu_B oxygen binding site. Therefore, CL may perturb the conformation of cytochrome *c* oxidase facilitating such electron transfers, or may increase the known conformational flexibility of the enzyme.

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