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Cardiolipin-Depleted Bovine Heart Cytochrome *c* Oxidase: Binding Stoichiometry and Affinity for Cardiolipin Derivatives†

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ABSTRACT: Detergent-solubilized bovine heart cytochrome *c* oxidase requires 2 mol of tightly bound cardiolipin (CL) per mole of monomeric complex for functional activity. Four lines of evidence support this conclusion: (1) Phospholipid depletion shows that two tightly bound CL's must remain associated with cytochrome *c* oxidase in order to maintain full electron transport activity. (2) Removal of the two tightly bound CL's correlates with decreased activity that is restored by reassociation of 2 mol of exogenous CL. (3) CL-depleted cytochrome *c* oxidase has two high-affinity binding sites for 2-[^{14}C]acetylcardiolipin (AcCL), $K_{d,app} < 0.1$ μM, that are not present in enzyme containing endogenous CL. An additional 2–3 lower affinity AcCL binding sites, $K_{d,app} = 4$ μM, are present in the CL-depleted complex, but these sites are also present in enzyme containing endogenous CL. (4) CL, monolysocardiolipin (MLCL), and dilysocardiolipin (DLCL) compete for AcCL binding with approximately the same relative affinities as those measured by the restoration of electron transport activity (MLCL competes much better than DLCL). However, MLCL and DLCL are only 60% and 15% as effective as CL in restoring maximum activity when they are bound to the high-affinity sites. The binding specificity of CL, MLCL, DLCL, and some of their acylated derivatives indicates that the apolar tails are most important for binding, not the polar head group. The presence or absence of hydroxyl groups in CL, MLCL, or DLCL also has little effect upon binding affinities. Binding specificity clearly favors CL since phosphatidylglycerol, phosphatidic acid, and phosphatidylcholine each have very low affinity for the CL binding sites ($K_{d,app} > 20$ μM). We, therefore, conclude that restoration of activity to CL-depleted cytochrome *c* oxidase is highly specific and requires the reassociation of CL, or structurally similar compounds, with two high-affinity binding sites.

Bovine cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase; EC 1.9.3.1) is the terminal enzyme complex of the inner mitochondrial electron transport chain and catalyzes electron transfer from reduced cytochrome *c* to molecular oxygen. The multisubunit enzyme complex spans the inner mitochondrial membrane and is in contact with the membrane phospholipids. Some of these mitochondrial membrane phospholipids copurify with cytochrome *c* oxidase, and the purified complex commonly contains 20–60 mol of PL[1]/mol of enzyme, i.e., bound phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL). PC and PE are easily extracted, or exchanged for detergent (Robinson & Capaldi, 1977; Robinson et al., 1980; Yu et al., 1975; Vik & Capaldi, 1977), but CL is tightly associated and is more difficult to remove.

Several studies have shown that the tightly associated CL is required for maximum electron transport activity of the bovine heart enzyme (Awasthi et al., 1971; Robinson et al., 1980; Fry & Green, 1980). When CL is removed, the resulting CL-depleted enzyme has only 30–50% of the original activity. Recovery of the remaining 50–70% activity is specific for CL and cannot be accomplished by other PL's (Robinson et al., 1980; Robinson, 1982; Dale & Robinson, 1988a). A number of reports support this observation although other PL's have been reported to restore activity to the CL-depleted enzyme (Awasthi et al., 1971; Fry & Green, 1980; Vik et al., 1981). Also, adriamycin, which specifically binds CL, inhibits cytochrome *c* oxidase and other CL-dependent enzymes (Goormaghtigh et al., 1982; Goormaghtigh & Ruysschaert, 1984). Some studies, however, indicate cytochrome *c* oxidase has no

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[1] Abbreviations: DM, dodecyl β-D-maltopyranoside; TX, Triton X-100; MA, molecular activity in μmol of ferrocytochrome *c* oxidized (μmol of cytochrome *c* oxidase)$^{-1}$ s^{-1}; Tris-HCl buffer, tris(hydroxymethyl)-aminomethane base titrated to the appropriate pH with hydrochloric acid; AcCL, 2-acetylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; Ac_2MLCL, diacetylated monolysocardiolipin or 1-(3-*sn*-phosphatidyl)-3-(2′-acetyl-3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; Ac_3-DLCL, triacetylated dilysocardiolipin or 1,3-bis(2′-acetyl-3-*sn*-phosphatidyl)-2-acetyl-*sn*-glycerol; CL, cardiolipin; MLCL, monolysocardiolipin; DLCL, dilysocardiolipin; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SucCL, 2-succinylcardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-succinyl-*sn*-glycerol; THP-CL, 2-(tetrahydropyranyl)cardiolipin or 1,3-bis(3-*sn*-phosphatidyl)-2-(tetrahydropyranyl)-*sn*-glycerol.

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special requirement for CL (Yu et al., 1975; Watts et al., 1978; Al-Tai et al., 1983; Thompson & Ferguson-Miller, 1983; Marsh & Powell, 1988). Whether or not CL has a functional role, CL is known to specifically interact with cytochrome *c* oxidase. Spin-labeled derivatives of CL preferentially bind to the CL-depleted enzyme (Cable & Powell, 1980; Knowles et al, 1981; Powell et al., 1985, 1987), and chemically reactive derivatives of CL preferentially react with some of the cytochrome *c* oxidase subunits (Kuppe et al., 1987; Dale & Robinson, 1988b; Fowler et al., 1988). The reason for the inconsistencies regarding the functional role of CL is not clear, but the widely different experimental approaches and the different sources of enzyme, e.g., yeast, dogfish, bovine liver, and bovine heart, as well as low estimates of CL content, may explain the different interpretations.

We have previously shown, with bovine heart cytochrome *c* oxidase, that there are 2–4 tightly bound CL's, which are essential for maximal electron transport activity. These can be exchanged for detergent at mildly alkaline pH if very high concentrations of detergent are used (Robinson et al., 1980; Robinson & Wiginton, 1985). The resulting CL-depleting enzyme is 30–40% active, but nearly full activity can be restored by association of exogenous CL with the CL-depleted complex. The functional binding affinities of CL, of several synthetic derivatives of CL, and of a number of other PL's have been determined (Robinson, 1982; Dale & Robinson, 1988a). On the basis of these studies, we concluded that restoration of full electron transport activity is specific for CL and that no other PL can restore activity. We also concluded that the apparent CL binding affinity, $K_{d,app}$, decreases when two fatty acyl groups are removed, or when they are replaced by shorter fatty acids.

In these previous studies, binding affinities were indirectly estimated, i.e., measured from the dependence of restoration of activity upon detergent-solubilized PL concentrations. By this approach, it is impossible to determine binding stoichiometry. For PL's that do not restore activity, kinetic studies also cannot distinguish between two very different possibilities: these ineffective PL's bind to the CL sites but do not affect activity, or they do not bind. In the present study, we show that functional kinetic studies do, in fact, reflect actual PL binding to the high-affinity CL binding sites. Because ^{14}C-acetylated CL restores activity to CL-depleted cytochrome *c* oxidase nearly as well as CL, it could be used to measure the binding of a functionally important phospholipid to the CL-depleted complex. Therefore, we were able to directly measure the stoichiometry and binding affinity of AcCL and, by appropriate competition experiments, to indirectly measure the stoichiometry and binding affinity of CL, its lyso derivatives, and two other acidic PL's.

Experimental Procedures

Materials

Cytochrome *c* oxidase was prepared from Keilin–Hartree heart muscle particles (Yonetani, 1960) by the method of Fowler et al. (1962) as modified by Capaldi and Hayashi (1972). The purified enzyme contained 9.1–9.3 nmol of heme A/mg of protein and had a molecular activity of 320–340 μmol of cytochrome *c* oxidized s^{-1} (μmol of cytochrome $aa_3)^{-1}$ when assayed in 0.025 M phosphate buffer, pH 7.0, containing either 2–10 mM dodecyl maltoside or 4 mM Tween 80. Chemicals were obtained from the following sources: CL, monolyso-CL (MLCL), and dilyso-CL (DLCL), in chloroform and under nitrogen, from Avanti Polar Lipids; [1-^{14}C]acetic anhydride (27.9 mCi/mmol) from Amersham; 4-(*N*,*N*-dimethylamino)pyridine and *N*,*N*-dicyclohexylcarbodiimide from Aldrich Chemical Co.; grade IV alumina from Woelm; specially purified Triton X-100 from Boehringer Mannheim; dodecyl maltoside from Calbiochem; Ultrapure enzyme-grade Tris base and Ultrapure redistilled glycerol from Bethesda Research Laboratories; type IV cytochrome *c* from Sigma; and DE-53 from Whatman.

Methods

Cytochrome *c* oxidase activity was measured spectrophotometrically by following the oxidation of 30 μM ferrocytochrome *c* in 0.7 mL of 0.025 M phosphate buffer containing either 10 mM dodecyl maltoside or 4 mM Tween 80, at pH 7.0 and 25 °C. Data were collected with an Adalab A/D converter and Apple II+ microcomputer; molecular activities were calculated from the first-order rate as described by Dale and Robinson (1988a). Restoration of activity to CL-depleted oxidase by exogenous CL was done as follows: cytochrome *c* oxidase (0.25 μM) was preincubated with 20–70 μM CL in 0.02 M Tris, 20% glycerol, 0.09 M NaCl, and 1 mM EDTA with 8 mM Triton X-100 at pH 8.0 on ice for 5 min (longer times did not change the activity). CL stock solutions were made up in this buffer with mild sonication immediately before use. The spectral assay was initiated by addition of 5 μL of the cytochrome *c* oxidase–CL solution to the 0.7-mL assay solution. Cytochrome *c* oxidase concentrations were calculated on the basis of $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1} (van Gelder, 1978).

Synthesis of Chemical Derivatives of Cardiolipin. Acetylated CL (AcCL), diacetylated MLCL (Ac_2MLCL), and triacetylated DLCL (Ac_3DLCL) were each prepared by acetylating 10 mM CL, MLCL, or DLCL, respectively, for 26 h with acetic anhydride (100 mM) and dicyclohexylcarbodiimide (20 mM), in 0.5 mL of dry, alumina-purified CH_2Cl_2 with 4-(*N*,*N*-dimethylamino)pyridine (20 mM) as a catalyst. Succinyl-CL (SucCL) was prepared by the same method except that succinic anhydride replaced acetic anhydride. [^{14}C]AcCL was also prepared identically except that 250 μCi (9 μmol) of [1-^{14}C]acetic anhydride (27.0 μCi/μmol) was added together with 40 μmol of the unlabeled acetic anhydride to the 0.5-mL reaction mixture. The specific activity of the final [^{14}C]AcCL was 2.56 μCi/μmol. Each of these acetylated or succinylated products was purified by (1) phase extraction of the phospholipid product according to Bligh and Dyer (1959); (2) washing the CH_2Cl_2 layer twice with Folch upper phase; (3) partial purification of the compound on alumina (Woelm N, activity grade I) by elution in chloroform/ethanol/50 mM ammonium acetate (18/25/7 v/v/v) after washing successively with chloroform, chloroform/methanol/water (66/33/3.2 v/v/v), and chloroform/methanol/water (10/10/3 v/v/v) (Luthra & Sheltawy, 1972); and (4) final purification of the product by isocratic HPLC at room temperature on a Waters Radial Pak Resolve silica 5-μm cartridge (0.8 × 10 cm) with cyclohexane/2-propanol/aqueous 10 mM H_3PO_4 (50/50/3.5 v/v/v) as the eluant at 1 mL/min (Robinson, 1990; Dale & Robinson, 1988a). Selective chemical modification of the 2-hydroxyl in CL and the 2- and 2′-hydroxyl(s) in MLCL and DLCL was confirmed by xenon fast atom bombardment mass spectra analysis of the synthetic CL analogues from a thioglycerol matrix with negative ion detection. The observed $[M_r - H]^-$ values (rounded to the nearest integer) and the expected $[M_r - H]^-$ values in parentheses), assuming $C_{18:2}$ acyl groups, for each compound are as follows: CL, 1448 (1447.9); MLCL, 1186 (1185.7); DLCL, 924 (923.5); AcCL, 1490 (1489.9); Ac_2MLCL, 1270 (1269.7); Ac_3DLCL, 1050 (1049.5); and SucCL, 1548 (1547.9). CL

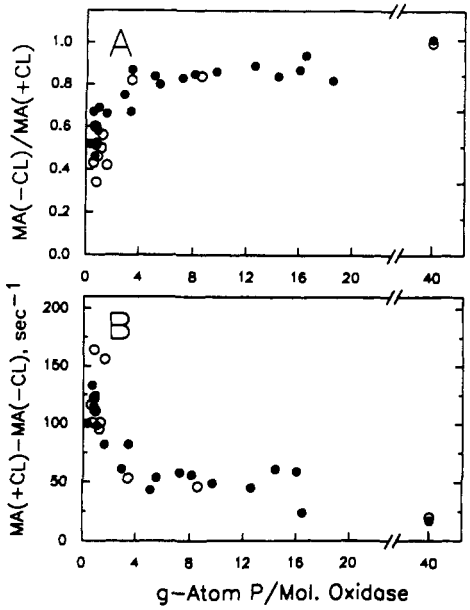

FIGURE 1: Effect of delipidation upon activity of cytochrome *c* oxidase. Samples were delipidated by glycerol gradient centrifugation in Triton X-100 as described under Methods. The amount of Triton X-100, pH, and ionic strength were each varied to give preparations with different amounts of phospholipid. Each point represents a separate glycerol gradient preparation of delipidated enzyme. Filled circles (●): Enzyme assays were done in 4 mM Tween 80. Open circles (○): Enzyme assays were done in 10 mM dodecyl maltoside. Panel A: Ratio of the molecular activity before addition of CL [MA(−CL)] to the molecular activity after addition of 20–70 μM CL [MA(+CL)]. No restoration of activity by CL would give a value of 1.0; a 3-fold increase in activity after addition of CL would give a value of 0.33. Panel B: Increase in molecular activity caused by the addition of 20–70 μM CL. For example, the data point with the greatest increase of 170 s^{-1} was obtained with enzyme that had an initial activity of 100 s^{-1} and a final activity of 270 s^{-1}. Each data point in panel A corresponds to a data point in panel B; therefore, for these same data, the ratio of the molecular activity before and after addition of CL is 0.37.

and the synthetic derivatives were stored under nitrogen or argon at −70 °C.

Phospholipid Depletion of Cytochrome c Oxidase. All of the phosphatidylcholine and phosphatidylethanolamine, but very little of the bound cardiolipin, was removed from the cytochrome *c* oxidase by (1) incubation of purified bovine cytochrome *c* oxidase (25 μM, 40 mol of PL/mol of oxidase) in pH 8.0, I = 0.1 buffer (0.02 M Tris, 0.09 M NaCl, 1 mM EDTA, 5% glycerol) containing 10 mg/mL Triton X-100 and (2) isolation of the delipidated complex by centrifugation through a discontinuous glycerol gradient (10%, 20%, 30%, and 50% glycerol) in pH 8.0 buffer containing 1 mg/mL Triton X-100 in either a 50 Ti (48 000 rpm, 16–18 h) or 50.2 Ti rotor (42 000 rpm, 16–20 h) [similar to Robinson et al. (1980)]. These preparations of mildly delipidated cytochrome *c* oxidase contained more than 8 mol of P/mol of complex, i.e., all of the tightly bound CL (3–4 mol of CL/mol of complex), and retained all of the electron transport activity of the original enzyme, 320–360 s^{-1}, when assayed in dodecyl maltoside or Tween 80 (refer to Figure 1A). If the ionic strength and/or pH were increased during a 4–5-h incubation in 50 mg/mL Triton X-100 (I = 0.5 or 1.0; pH 8.5, 9.0, or 10.0), the complex was depleted of CL (only 0.5–8 mol of P/mol of complex, i.e., 0.25–4 mol of CL/mol of complex, depending upon the incubation conditions) when it was isolated by a similar discontinuous glycerol gradient at pH 8.0 with 10 mg/mL Triton X-100 (Robinson & Wiginton, 1985). These CL-depleted preparations had decreased electron transport activity, as low as 90 s^{-1}, when assayed in dodecyl maltoside or Tween 80 (refer to Figure 1A). In samples containing 1 M NaCl, 1 M NaCl was included in the 10% glycerol layer of the gradient and 0.5 M NaCl in the 20% glycerol layer to prevent the sample from sinking through the gradient; in samples containing 0.5 M NaCl, 0.5 M NaCl was included in the 10% glycerol layer for the same reason. The buffers used for the Triton X-100 incubation were as follows: (1) I = 0.1 buffers: pH 8.0 and 8.5 (0.2 M Tris, 0.1 mM EDTA); pH 9.0 (0.2 M glycine, 0.16 M NaCl, 0.1 mM EDTA); pH 10.0 (0.2 M glycine, 0.04 M NaCl, 0.1 mM EDTA). (2) I = 0.5 buffers included an additional 0.4 M NaCl; I = 1.0 buffers included an additional 0.9 M NaCl.

Phospholipid Determinations. Concentrations were determined by a modification of the phosphate method of Chen et al. (1956) after digestion in 0.5 mL of perchloric acid according to the procedure of Marinetti (1962). The phosphorus content of delipidated cytochrome *c* oxidase preparations was determined by the same assay procedure after removing the large amount of Triton X-100 from 4 nmol of delipidated cytochrome *c* oxidase by either (1) lyophilization of sample, extraction of Triton X-100 with cold, dry acetone, and digestion of the protein residue with perchloric acid or (2) extraction of phospholipids and Triton X-100 by the method of Bligh and Dyer (1959), streaking the $CHCl_3$-containing phospholipids and Triton X-100 at the bottom of an acetone-washed TLC plate, developing the plate in dry acetone to extract Triton X-100, and scraping 10 cm^2 of the silica gel at the bottom into the perchloric acid digestion tube for determination of total P. Delipidated cytochrome *c* oxidase samples were usually analyzed for P by both methods. The total digest of the lyophilized, acetone-extracted sample sometimes gave a slightly higher P content than analysis of the Bligh and Dyer extracted phospholipid, e.g., 0.9 compared with 0.4 mol of P/mol of complex, which could be due to either small amounts of inorganic phosphate in the sample or incomplete extraction of the tightly bound CL by the Bligh and Dyer procedure. Each value in Figure 1 is the average obtained by both methods, and these never differed by more than 0.5 mol of P/mol of complex. For accurate phospholipid determinations (±0.2 mol of P/mol of complex), Triton X-100 must be P free. Specially purified Triton X-100 obtained from Boehringer Mannheim contained less than 0.05 nmol of P/mg, but Triton X-100 from Research Products International contained 2 nmol of P/mg of detergent. When this source of Triton X-100 was used in some of the earlier experiments, the phosphate contaminants were removed by phase extraction of the Triton X-100 (200 g) from 1.5 L of chloroform/methanol/aqueous 0.1 M NaCl (1/1/0.9 v/v/v), removal of the chloroform with a rotovap, and then high vacuum. P content, after phase extraction, was reduced to less than 0.05 nmol/mg.

Subunit Analysis of Delipidated Oxidase. Most CL-depleted preparations of cytochrome *c* oxidase were depleted of subunits VIa and VIb, and those treated with high salt and pH >9 were also at least partially depleted of subunit III (Robinson & Wiginton, 1985). Subunit compositions were determined by (1) polyacrylamide gel electrophoresis in sodium dodecyl sulfate and 6 M urea according to Kadenbach et al. (1983), in sodium dodecyl sulfate and 2 M urea as previously described (Robinson et al., 1980), and (2) HPLC acetonitrile gradient elution from a C-4 reverse-phase Vydac column in 0.1% TFA (Robinson et al., 1990) after exchanging dodecyl maltoside for Triton X-100 by DEAE ion-exchange chromatography.

[^{14}C]Acetylcardiolipin Binding to Cytochrome c Oxidase. Cytochrome *c* oxidase (1.25 μM) was incubated with 0–10 μM

[^{14}C]AcCL at 0 °C in 0.5 mL of 10 mM Tris buffer, pH 8, containing 45 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 4.7 mM Triton X-100 (40 μM micelles), i.e., 0–0.25 mol of [^{14}C]AcCL/mol of micelles. After 5 min the sample was applied to a 0.58 × 3 cm DE-53 column equilibrated with the pH 8 buffer to separate the cytochrome c oxidase–[^{14}C]AcCL complex from detergent-solubilized, unbound [^{14}C]AcCL. After the column was washed with 5–6 mL of buffer, the complex was eluted with buffer containing 0.45 M NaCl. The concentration of cytochrome c oxidase in each 0.55-mL fraction was determined by assaying a 100-μL aliquot for protein with the Pierce BCA protein assay; the concentration of [^{14}C]AcCL was determined by scintillation counting of a 250-μL aliquot. All of the [^{14}C]AcCL that coeluted with cytochrome c oxidase was assumed to be bound. The free concentration in the original incubation mixture was calculated by subtracting the total [^{14}C]AcCL that was bound from the total added. Radioactivity was measured in a Tracor Analytic Model 6892 scintillation counter in 5 mL of toluene/Triton X-100 scintillation liquid: toluene (2 L), Triton X-100 (1 L), 2,5-diphenyloxazole (PPO, 8 g), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP, 0.4 g).

Results

Two Cardiolipins Are Required for Full Electron Transport Activity. The electron transport activity of more than 30 Triton X-100 delipidated preparations of cytochrome c oxidase was analyzed as a function of phospholipid content. Preparations of delipidated cytochrome c oxidase that had more than 4 mol of P/mol of complex had approximately the same electron transport activity as the original preparation containing 40 mol of P/mol of complex. In those preparations with less than 4 mol of P/mol of complex, phospholipid removal caused a decrease in activity that could be restored by incubation with low concentrations of exogenous CL dissolved in detergent micelles (<1 CL/micelle) (Figure 1). In samples containing less than 6 mol of P/mol of complex, all of the P is due to tightly bound CL (Robinson et al., 1980). In samples with the least amount of tightly bound CL (less than 1 CL/complex, i.e., <2 P/complex; MA = 90–100 s^{-1}) incubation with CL increases the activity by as much as 150–170 s^{-1} (Figure 1B); i.e., the final activity after incubation increased 2–3-fold and was about 80% of the activity prior to delipidation. The 20% activity which could not be restored by CL was probably lost due to nonspecific denaturation of cytochrome c oxidase during the fairly harsh delipidation procedures. In samples containing more than 4 P/complex, i.e., more than 2 CL/complex, the complex always contained both tightly bound endogenous CL's and had an activity of 280–340 s^{-1}. Incubation of these samples with detergent-solubilized CL had little effect on the activity, i.e., MA(−CL)/MA(+CL) > 0.8–0.9 (Figure 1A) and MA(+CL) − MA(−CL) = 40–50^{-1} (Figure 1B). Since exogenous CL significantly stimulates only those delipidated samples containing less than 2 CL/complex, we have concluded that maximal electron transport activity of bovine cytochrome c oxidase has a functional requirement for 2 tightly bound CL's.

Measurement of Functional Binding Affinity of Cardiolipin and Its Derivatives. Addition of exogenous CL to CL-depleted cytochrome c oxidase restores activity in a concentration-dependent manner that permits evaluation of the apparent dissociation constant ($K_{d,app}$) for functionally important CL (Robinson, 1982; Dale & Robinson, 1988a). In the present study, $K_{d,app}$ and maximum restored activity (ΔMA_{max}) were measured for CL, MLCL, DLCL, AcCL, Ac_2MLCL, Ac_3DLCL, SucCL, phosphatidic acid, and phosphatidylglycerol.

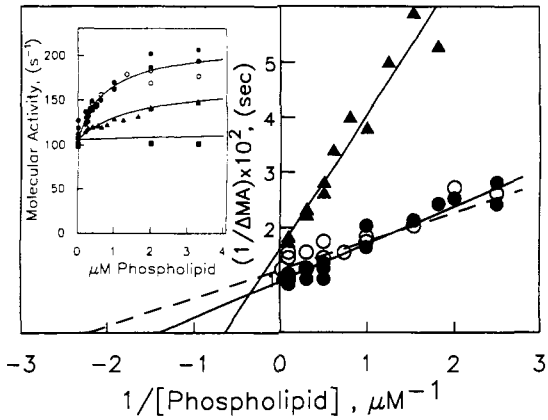

FIGURE 2: Kinetics of the restoration of activity to cardiolipin-depleted cytochrome c oxidase by cardiolipin and cardiolipin derivatives. In both graphs, filled circles (●) are CL data open circles (○) are AcCL data; and filled triangles (▲) are MLCL data. In the inset the filled squares (■) are DLCL data. Inset: Measured molecular activity as a function of added CL or CL derivative concentration. Lines represent the best fit to the data with the $K_{d,app}$ and ΔMA_{max} values in Table I. The double-reciprocal plot is for the same data, illustrating the linearity of the reciprocal of the change in activity (ΔMA) as a function of the reciprocal of the CL, or CL derivative, concentration. The two solid lines are the best fit to CL and MLCL data; the dashed line is the best fit to AcCL data.

Table I: Restoration of Activity by Derivatives of Cardiolipin[a]

phospholipid	ΔMA_{max}	$K_{d,app}$
CL	106 ± 4	0.7 ± 0.1
AcCL	89 ± 4	0.4 ± 0.1
SucCL	92 ± 4	0.5 ± 0.1
MLCL	65 ± 2	1.7 ± 0.4
Ac_2MLCL	63 ± 2	2.2 ± 0.7
DLCL	15 ± 15	>20
Ac_3DLCL	15 ± 15	>20
PA	60 ± 20	>20
PG	70 ± 25	>20

[a] CL-depleted cytochrome c oxidase (0.5 CL/complex), in 40 μM Triton X-100 micelles, was incubated with 0–10 μM phospholipid, and ΔMA_{max} and $K_{d,app}$ were evaluated by fitting both the hyperbolic stimulation curve with Enzfitter (inset Figure 2) and the double-reciprocal plot with Quattro (Figure 2). Agreement by the two methods was good, and the values listed are the averages except for the derivatives that had very low affinity and almost no restoration of activity, e.g., Ac_3DLCL and DLCL. In these two cases it was impossible to fit a hyperbola, and the constants were evaluated from the double-reciprocal plot. Errors are those calculated by the fitting programs.

The inset in Figure 2 illustrates the restoration of activity to CL-depleted oxidase (0.5 mol of CL/mol of complex; MA = 100 s^{-1}) by CL, MLCL, and AcCL. The linear double-reciprocal plot in Figure 2 confirms the hyperbolic response of the restoration of activity by added cardiolipin or its derivatives. Nearly identical values were obtained for the apparent dissociation constant ($K_{d,app}$) and the maximum restored activity (ΔMA_{max}) when they were evaluated by fitting the data in either the hyperbolic plot or the double-reciprocal plot. Values for $K_{d,app}$ and ΔMA_{max} for each of the cardiolipin analogues are summarized in Table I. Clearly, modification of the hydroxyl on the bridge glycerol of CL has very little effect on either the $K_{d,app}$ or ΔMA_{max}; i.e., CL, AcCL, and SucCL bind and stimulate the activity almost identically, as do MLCL and Ac_2MLCL, and also DLCL and Ac_3DLCL. Addition of 5 mM Ca^{2+} during the CL incubation had no effect upon the restoration of activity.

Direct Measurement of [^{14}C]Acetylcardiolipin Binding. Because CL and AcCL are nearly equal in restoring activity to CL-depleted cytochrome c oxidase, the dissociation constant and maximum binding of CL can be estimated by measuring

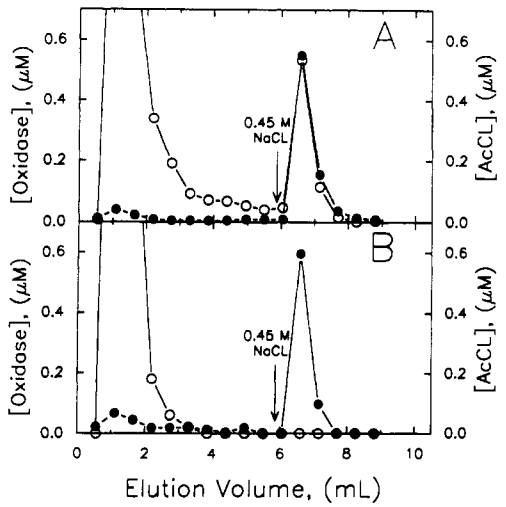

FIGURE 3: DEAE column binding assay. In both panels, open circles (O) are [^{14}C]AcCL concentrations; filled circles (●) are cytochrome *c* oxidase concentrations. Panel A is representative of a typical binding assay in which 0.95 mol of [^{14}C]AcCL was bound per mole of cytochrome *c* oxidase; AcCL was incubated for 5 min with CL-depleted cytochrome *c* oxidase, and then the AcCL complex was separated from free CL by batch elution from a 0.58 × 3 cm DE-53 column. Panel B is a control in which the [^{14}C]AcCL and cytochrome *c* oxidase were each applied and run separately; note that no [^{14}C]AcCL elutes with 0.45 M NaCl.

the binding of the synthetic CL derivative, [^{14}C]AcCL. CL-depleted cytochrome *c* oxidase (0.5 mol of CL/mol of complex) was incubated with 0–25 μM [^{14}C]AcCL dissolved in 4.7 mM Triton X-100 (40 μM micelles); unbound AcCL was separated from cytochrome *c* oxidase bound AcCL by a small DEAE ion-exchange column (Figure 3). [^{14}C]AcCL that comigrated with cytochrome *c* oxidase when it was eluted from the column with 0.45 M NaCl was considered to be bound. The free concentration of [^{14}C]AcCL in equilibrium with the AcCL–oxidase complex was estimated by subtracting the bound [^{14}C]AcCL from the total [^{14}C]AcCL that was initially added. In this way, the binding of [^{14}C]AcCL as a function of [AcCL] was determined for CL-depleted cytochrome *c* oxidase and for enzyme that contained a full complement of endogenous CL, i.e., nondelipidated oxidase (Figure 4). Binding to the CL-depleted enzyme was biphasic and could not be fitted with a single hyperbolic curve. The biphasic binding curve could be fitted reasonably well by the sum of two hyperbolic binding curves: high-affinity binding of 1.5–2.0 AcCL/oxidase between 0 and 0.1 μM AcCL followed by the additional nonspecific binding of 2–3 AcCL between 0.1 and 25 μM AcCL. Nonspecific binding of [^{14}C]AcCL to the CL-depleted and nondelipidated preparations of cytochrome *c* oxidase was very similar since both of the curves in Figure 4 are nearly parallel over the entire range of [^{14}C]AcCL concentrations. However, binding to the CL-depleted preparation was always greater by 1.5–2.0 mol of [^{14}C]AcCL/mol of complex, a value that represents the stoichiometry of the high-affinity CL binding sites on CL-depleted enzyme (inset to Figure 4). The dissociation constant and maximum binding of [^{14}C]AcCL to the nonspecific binding sites was evaluated from the data obtained with nondelipidated cytochrome *c* oxidase; the dissociation constant and maximum binding to the high-affinity binding sites on CL-depleted cytochrome *c* oxidase were estimated to be less than 0.04 μM after appropriate correcting for nonspecific binding (refer to the inset in Figure 4). Values for $K_{d,app}$ and ν_{max} for both the high-affinity and nonspecific binding sites are summarized in Table II. Notice that the maximum binding together with the amount of endogenous

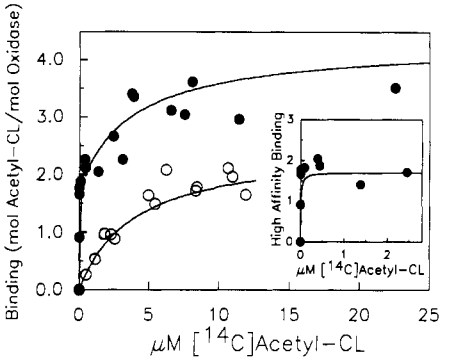

FIGURE 4: Binding of [^{14}C]AcCL to CL-depleted and nondelipidated cytochrome *c* oxidase. In each binding experiment, the [^{14}C]-AcCL–oxidase complex was separated from free [^{14}C]AcCL by the DEAE column binding assay as illustrated in Figure 3. Delipidated oxidase, filled circles (●), contained less than 0.5 mol of CL/mol of cytochrome *c* oxidase; nondelipidated oxidase, open circles (O), contained 40 mol of P/mol of cytochrome *c* oxidase, of which 20% was contributed by CL. The two solid lines represent theoretical binding curves to each form of oxidase; these were generated with the $K_{d,1}$, $\nu_{max,1}$, $K_{d,2}$, and $\nu_{max,2}$ values for AcCL given in Table II and eq 1 below, which assumes two types of independent, noninteracting binding sites. Inset: Specific binding of [^{14}C]AcCL to the high-affinity sites (mol of AcCL/mol of oxidase). Specific binding to the high-affinity sites, filled circles (●), was calculated by subtracting the nonspecific binding (theoretical curve for binding of [^{14}C]AcCL binding to nondelipidated enzyme in main figure) from the observed binding to the CL-depleted enzyme. The line through the data is a theoretical line calculated from the specific binding constants in Table II. The equation used for calculation of theoretical binding curves in the main figure is eq 1, where $\nu_{max,1,AcCL}$ = maximum binding of

$$\nu_{obs} = \frac{\nu_{max,1,AcCL}[AcCL]}{K_{d,1,AcCL} + [AcCL]} + \frac{\nu_{max,2,AcCL}[AcCL]}{K_{d,2,AcCL} + [AcCL]} \quad (1)$$

AcCL to high-affinity sites, $K_{d,1,AcCL} = K_{d,app}$ of AcCL to high-affinity sites, $\nu_{max,2,AcCL}$ = maximum binding of AcCL to low-affinity sites, and $K_{d,2,AcCL} = K_{d,app}$ of AcCL to low-affinity sites.

Table II: Binding Constants for Acetylcardiolipin, Cardiolipin, Monolysocardiolipin, and Dilysocardiolipin Binding to Cardiolipin-Depleted Cytochrome *c* Oxidase[a]

phospholipid	$K_{d,1}$ (μM)	$\nu_{max,1}$ (mol of PL/mol of complex)	$K_{d,2}$ (μM)	$\nu_{max,2}$ (mol of PL/mol of 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] In each experiment 1.15 μM cytochrome *c* oxidase was incubated in 0.1 M Tris-HCl buffer, pH 7.8, containing 4.7 mM Triton X-100 (40 μM micelles) with 10 μM [^{14}C]AcCL in the presence of 0–90 μM of the competing unlabeled phospholipid (refer to Figure 5). Binding was measured after separation of cytochrome *c* oxidase containing the bound AcCL on a DEAE column as described under Methods. Data listed in the table for $K_{d,1}$, $K_{d,2}$, $\nu_{max,1}$, and $\nu_{max,2}$ correspond to values used to generate the theoretical binding curves shown in Figure 5 with the equation described in the legend to this figure. Absolute values for these binding constants can only be estimated since the free concentration of AcCL during saturation of the high-affinity sites is too low to be calculated by subtracting bound [^{14}C]AcCL from the total added. The relative values, however, are much more accurate since the binding constants for AcCL:CL:MLCL:DLCL must be close to 1:2:10:100 to fit the competition data in Figure 5.

CL (0.5 mol of CL/mol of complex) is nearly identical with our estimate of 2 tightly bound CL's (Figure 1). The $K_{d,app}$ obtained from the binding measurements is also about 10-fold smaller than the value obtained from kinetic data (Table I). This may be due to the fact that the concentration of AcCL in the incubation mixture was used to calculate $K_{d,app}$ in the

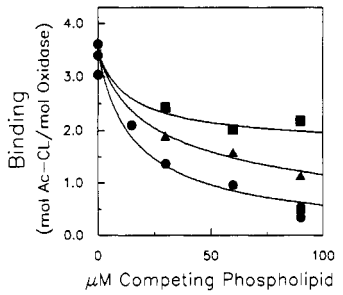

FIGURE 5: Competition of unlabeled CL and CL derivatives for [^{14}C]AcCL binding to CL-depleted cytochrome *c* oxidase. The competing PL's were as follows: CL, filled circles (●); MLCL, filled triangles (▲); and DLCL, filled squares (■). In each experiment, 10 μM [^{14}C]AcCL was added to 1.2 μM enzyme in the presence of the indicated amount of competing PL. In the absence of any competing PL, 3.5 mol of [^{14}C]AcCL bound per mole of enzyme. The theoretical lines drawn through the data are based upon the following assumptions: (1) [^{14}C]AcCL can bind to two types of independent noninteracting sites on CL-depleted cytochrome *c* oxidase, as described in the legend to Figure 4; (2) unlabeled CL, MLCL, and DLCL exhibit classical competition for [^{14}C]AcCL binding at both the low- and high-affinity binding sites; i.e., total binding can be represented by eq 2, where $\nu_{max,1,AcCL}$, $\nu_{max,2,AcCL}$, $K_{d,1,AcCL}$, and $K_{d,2,AcCL}$ are defined

$$\nu_{obs} = \frac{\nu_{max,1,AcCL}[AcCL]}{K_{d,1,AcCL}(1 + K_{d,1,PL}/[PL]) + [AcCL]} + \frac{\nu_{max,2,AcCL}[AcCL]}{K_{d,2,AcCL}(1 + K_{d,2,PL}/[PL]) + [AcCL]} \quad (2)$$

in the legend to Figure 4, $K_{d,1,PL} = K_{d,app}$ of competing PL to high-affinity sites, and $K_{d,2,PL} = K_{d,app}$ of PL to low-affinity sites; (3) binding of [^{14}C]AcCL to CL-depleted cytochrome *c* oxidase has a $K_{d,1,app}$ = 0.04 μM and $\nu_{max,1}$ = 1.8 mol of [^{14}C]AcCL/mol of complex for the high-affinity sites and a $K_{d,2,app}$ = 4 μM and $\nu_{max,2}$ = 2.5 mol of [^{14}C]AcCL/mol of complex (these are the values in Table II, which are derived from the binding data in Figure 4); (4) the stoichiometry of CL, MLCL, and DLCL binding to the high-affinity and low-affinity sites is the same as measured for [^{14}C]AcCL; (5) the relative binding affinities of AcCL, CL, MLCL, and DLCL to the high-affinity sites are the same as found in the functional binding studies (Table I); and (6) the binding affinity of CL, MLCL, and DLCL to the low-affinity sites is the same as for AcCL, i.e., 4 μM.

kinetic experiments and the 150-fold dilution into the enzyme assay was not included. It also could be due to underestimation of the free [^{14}C]AcCL concentration during the binding measurements since it was impossible to measure this concentration directly. Nevertheless, binding is at least as tight as previously determined from kinetic data.

Binding of [^{14}C]phosphatidylcholine to CL-depleted cytochrome *c* oxidase was also measured by the DE-53 binding method. With $[PC]_{free}$ = 10 μM, only 0.15 mol of PC was bound per mole of oxidase. Assuming the same 4 binding sites are available for PC as were available for AcCL, $K_{d,app}$ for PC would be greater than 200 μM.

Competition of Cardiolipin and Other Phospholipids for [^{14}C]AcCL Binding. The true binding constant for other phospholipids to CL-depleted cytochrome *c* oxidase was estimated by measuring their effectiveness in competing for [^{14}C]AcCL binding. CL-depleted oxidase (1.2 μM) was incubated with 10 μM [^{14}C]AcCL, resulting in 3.5 mol of AcCL bound per mole of complex. Increasing concentrations of unlabeled CL, MLCL, and DLCL (15–90 μM) were added to compete for the binding of labeled AcCL (Figure 5). Each of the phospholipids competed with [^{14}C]AcCL for binding to the CL enzyme; CL was the most effective, and DLCL was the least effective. The values for the dissociation constants, $K_{d,app}$, and maximum binding at the high-affinity sites, $\nu_{1,max}$, and low-affinity sites, $\nu_{2,max}$, of the competing PL's are summarized in Table II. Notice that $K_{d,app}$ values for CL, MLCL, and DLCL from these competition experiments are similar to the values obtained from the kinetic data (Table I), but about 10-fold smaller. Possible reasons for this 10-fold difference were mentioned in the last section.

Table III: Competition of Unlabeled Phospholipids for Binding of [^{14}C]Acetylcardiolipin to Cardiolipin-Depleted Cytochrome *c* Oxidase[a]

competing phospholipid	bound[b] (mol of AcCL/mol of complex)	free (mM AcCL)
none	1.8 ± 0.1	0.05
20 μM CL	0.3 ± 0.05	1.5
20 μM MLCL	0.7	1.0
20 μM DLCL	1.3	0.6
20 μM PA	1.1 ± 0.1	0.8
20 μM PG	1.4	0.3

[a] In each experiment 1.15 μM cytochrome *c* oxidase was incubated in 0.1 M Tris-HCl buffer, pH 7.8, containing 4.7 mM Triton X-100 (40 μM micelles) with 1.9–2.1 μM [^{14}C]AcCL in the presence of 20 μM of the competing unlabeled phospholipid. Binding was measured after separation of cytochrome *c* oxidase containing the bound AcCL on a DEAE column as described under Methods. [b] In experiments where error bars are included, experiments are the averages of duplicates or triplicates.

A second competition experiment was done after adding only 2 μM [^{14}C]AcCL where very little nonspecific binding occurs, i.e., 1.8 AcCL bound/complex with free [^{14}C]AcCL ≤ 0.1 μM. Fairly high concentrations of CL, MLCL, DLCL, phosphatidic acid, and phosphatidylglycerol (20 μM) were added to compete for binding of [^{14}C]AcCL (Table III). CL effectively competed and displaced 83% of the AcCL; MLCL and phosphatidic acid partially competed and displaced 60% and 50% of the AcCL, respectively; DLCL and phosphatidylglycerol were ineffective and displaced less 30% of the AcCL. On the basis of the data in Figure 4, perfect competition of a phospholipid with the same binding constant as AcCL would have resulted in displacement of 70% of the AcCL.

Discussion

The present study clearly shows that CL, AcCL, and MLCL restore electron transport activity to CL-depleted cytochrome *c* oxidase by specifically binding to two high-affinity CL sites on the enzyme. Presumably, other phospholipids that are known to restore activity, e.g., SucCL and Ac_2MLCL (present study) and tetrahydropyranyl-CL and acylated CL (Dale & Robinson, 1988a), also bind to these same sites. Lipid depletion, restoration of activity by CL and its derivatives, and direct binding studies provide evidence for this conclusion of a specific interaction of CL with cytochrome *c* oxidase.

First, both lipid depletion (Figure 1) and direct binding experiments (Figure 4 and Table II) give essentially the same CL stoichiometry for saturation of the essential high-affinity sites. In normal preparations of the beef heart enzyme, there are two tightly bound CL's that are difficult to remove; i.e., two sites are occupied by endogenous CL (refer to Figure 1; Robinson & Wiginton, 1985; Thompson & Ferguson-Miller, 1983). Extra PL (phosphatidylcholine, phosphatidylethanolamine, and CL) may or may not be present, but the presence or absence of extra PL has almost no effect on activity (refer to Figure 1). Enzyme that has endogenous CL occupying the two high-affinity CL sites is stimulated very little by addition of detergent-solubilized CL; however, removal of the two tightly bound endogenous CL's causes decreased activity that can be restored by addition of CL (refer to Figures 1 and 2). Both the [^{14}C]AcCL binding studies (Figure 4) and the CL competition data (Table II) indicate that 2 mol of [^{14}C]AcCL, CL, or MLCL specifically reassociates with the

CL-depleted enzyme, i.e., 1.8 mol binds to enzyme already containing 0.7 mol of endogenous CL. We conclude it is the reassociation of 2 mol of any of these PL's with CL-depleted cytochrome *c* oxidase that restores full activity.

Second, binding to the high-affinity sites is quite tight, considering 4–10 mM detergent is present in all of the experiments. Apparent binding constants estimated from fitting of kinetic data (Table I) also agree quite well with apparent binding constants calculated from the [^{14}C]AcCL binding and PL competition experiments (Table III). In each case, AcCL binds about the same as CL, about 3–10 times better than MLCL, and about 50–100 times better than DLCL. The relative binding constants, rather than absolute values, are the most important. In both types of experiments, dissociation constants were measured by nonequilibrium methods, and discrepancies in the values obtained by the two methods may not be as large as indicated in Tables I and II. The [^{14}C]AcCL binding assay probably gives a low estimate for $K_{d,app}$ (Table II) due to some release of bound label during DEAE chromatography (no [^{14}C]AcCL was present in the eluting buffer). Conversely, the stimulation of electron-transfer activity probably gives a high estimate for $K_{d,app}$ (Table I) due to dilution of the CL derivatives during the assay. The true dissociation constant of each phospholipid is probably between the values shown in these two tables. Even with this minor difficulty, it is possible to conclude that removal of fatty acyl groups from CL decreases its binding affinity and that removal of the second group affects binding more than removal of the first group.

Third, PL's that do not, or only poorly, restore activity to CL-depleted oxidase have very low affinity for the two high-affinity CL sites, i.e., DLCL, PA, and PG (Table II). They bind no better to the CL sites than they bind to the nonspecific sites (refer to Table II). In contrast to these lipids, CL binds more than 100 times tighter to the specific high-affinity sites.

Lastly, all of the PL's that were tested had a relatively low affinity for 2–3 nonspecific sites on the detergent-solubilized enzyme; the binding constant of [^{14}C]AcCL for these nonspecific sites on either the nondelipidated enzyme or the CL-depleted enzyme is at least 100-fold less than that of the specific sites on the CL-depleted enzyme. PL's that bind poorly to the high-affinity sites, e.g., DLCL, effectively displace CL from the nonspecific sites although they do not displace CL from the specific high-affinity sites (Figures 5 and Table II). Best estimates for the nonspecific binding of all of the PL's to the nonspecific sites is very similar, i.e., $K_{d,app} = 4\ \mu M$.

All of these experiments support the hypothesis that CL binds to a small number of sites on the surface of cytochrome *c* oxidase that are highly specific for CL. Since chemical modification of the hydroxyl on the bridge glycerol of CL with an acetyl or succinyl group (present study), or with an oleoyl acyl group (Dale & Robinson, 1988a), has little effect upon reassociation, it is likely that recognition and high affinity for CL is not dependent upon specific interactions with the bridge glycerol but is dependent upon the four fatty acyl tails. Results obtained here with MLCL and DLCL support this idea since removal of one acyl group decreases functional and real binding 3–10-fold and removal of two acyl groups decreases these same parameters 30–100-fold. The PA and PG results also suggest that they bind better than DLCL even though each is an acidic PL that contains two fatty acyl groups (these three PL's all bind so weakly that the K_d's are subject to large errors). Thus, binding affinity and specificity probably lie in recognition of the correct structure in the apolar region and not a generalized apolar interaction.

The high affinity and specificity of cytochrome *c* oxidase for CL that we detect by [^{14}C]AcCL binding are not in complete agreement with some past studies on this enzyme. The dogfish and rat liver enzymes, on the basis of the lack of CL in the purified enzyme, are reported not to require CL (Al-Tai et al., 1983; Thompson & Ferguson-Miller, 1983). Electron transport activity of the CL-depleted yeast enzyme, however, is stimulated by CL (Marsh & Powell, 1988), much like we find for the beef heart enzyme. In spite of general agreement that CL stimulates the activity of both the yeast and bovine enzymes, our conclusions regarding the high-affinity binding of CL and its analogues to the bovine enzyme are quite unlike the conclusions drawn from the immobilization of spin-labeled CL to the yeast and bovine enzymes (Knowles et al., 1981; Powell et al., 1985, 1987). Because the [^{14}C]AcCL binding studies and the spin label binding experiments are done quite differently, complete agreement may not be expected. The spin label experiments were interpreted in terms of a single class of binding sites in which CL bound to the entire apolar surface of cytochrome *c* oxidase, i.e., CL bound to about 50 sites/complex. High-affinity binding to a small number of CL-specific sites was not reported. This single class of low-affinity sites did exhibit a slight specificity for spin-labeled CL over other phospholipids; i.e., CL bound 3–5-fold stronger than PC. There are several important differences between the present study and these spin label experiments. First, [^{14}C]AcCL binding experiments allow the ligand concentration to be adjusted over a fairly wide range of concentrations; the spin label binding studies were all done at a single concentration of spin-labeled CL. Second, our binding studies are all done in the presence of a large excess of detergent (4000-fold molar excess of detergent over enzyme) which nearly prevents the binding of AcCL to nonspecific sites; the spin label studies were done with only a 100–500-fold molar excess of PC over enzyme, and therefore, nonspecific binding may be more prevalent. Third, we have measured the difference in AcCL binding to the CL-depleted and nondelipidated enzyme, which permits us to detect high-affinity, CL-specific binding; the spin label studies were done only on the CL-depleted enzyme. Fourth, the spin label binding experiments, with competition by PC, are the most similar to our competition binding experiment with DLCL (Figure 5). (We find that DLCL and PC both have low affinity for the CL-specific sites.) Because the competing PC concentration was only varied 5-fold molar in the spin label experiments (100–500 times the enzyme concentration), our data with 20–100 μM DLCL are the most comparable. In this concentration range we find that 70–75% of the added [^{14}C]AcCL is not bound, a result that is not significantly different from the spin label results. Lastly, the data obtained with spin-labeled CL are not inconsistent with a small number of higher affinity sites in addition to the 50 low-affinity sites. The amount of downward curvature in the binding study of Knowles et al. (1981) suggests that PC does not equilibrate with about half of the bound spin-labeled CL, indicative of a small number of CL-specific sites. In spite of these differences in detecting or not detecting high-affinity, CL-specific sites, both methods give approximately the same order for the specificity of binding of the various CL analogues; e.g., the binding affinity is CL > MLCL > PA > PC.

The present direct binding experiments certainly confirm our earlier suggestion that CL binds to detergent-solubilized, CL-depleted cytochrome *c* oxidase with (1) high affinity, (2) low stoichiometry, and (3) high specificity. *High affinity* is apparent by comparison of the AcCL binding constant (Table

II) to the micellar concentration of detergent. When 100% of the enzyme binds 2 mol of CL at the high-affinity sites, e.g., $[AcCL]_{free} = 0.1\ \mu M$, only 1–2% of the 40 μM micelles contain a single AcCL. CL clearly partitions from the detergent micellar environment to cytochrome *c* oxidase–detergent complex, and this transfer is highly favored. Binding to the nonspecific sites is much more random. For 2 AcCL to be bound to the nonspecific sites, e.g., $[AcCL]_{free} = 20\ \mu M$, 50% of the micelles would contain a single AcCL. Our results are, therefore, consistent with the nonspecific binding caused by random association of AcCL with detergent micelles and the cytochrome *c* oxidase–detergent complex. The *low binding stoichiometry* of the high-affinity sites, 2 CL's per cytochrome *c* oxidase, is quite low and unusual for the interaction of a PL with a membrane protein. Whether tightly bound CL has a specific catalytic role, is involved in the binding of cytochrome *c*, affects the transition from one cytochrome *c* oxidase conformer to another, or indirectly affects electron transport from cytochrome *a* to cytochrome a_3 is presently unknown. *High CL specificity* is not unique, and several other mitochondrial enzymes, translocases, and signal peptides have been reported to be specific for CL. Whether they will exhibit the same type of interaction with CL as we have shown with cytochrome *c* oxidase remains to be tested.

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Registry No. Cytochrome *c* oxidase, 9001-16-5.

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