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Phospholipase A_2 Digestion of Cardiolipin Bound to Bovine Cytochrome *c* Oxidase Alters Both Activity and Quaternary Structure†

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ABSTRACT: Phospholipase A_2 from *Crotalus atrox* hydrolyzes all of the phospholipids that are associated with purified, detergent-solubilized cytochrome *c* oxidase; less than 0.05 mol cardiolipin (CL)[1] remains bound per mol enzyme. Coincident with the hydrolysis of cardiolipin is a reversible decrease of 45−50% in the electron transport activity of the dodecylmaltoside-solubilized enzyme. Full activity is recoverable (90−98%) by addition of exogenous cardiolipin, but not by either phosphatidylcholine or phosphatidylethanolamine. Unexpectedly, cleavage of cardiolipin causes the dissociation of both subunits VIa and VIb from the enzyme. These are the two subunits that form the major protein−protein contacts between the two monomeric units within the dimeric complex. Although hydrolysis of CL by phospholipase A_2 and loss of these subunits is linked, the reverse process does not occur, i.e., removal of subunits VIa and VIb does not cause dissociation of the two functionally important, tightly bound cardiolipins. Nor does addition of exogenous cardiolipin result in reassociation of the two subunits with the remainder of the complex. We conclude that cardiolipin is not only essential for full electron transport activity, but also has an important structural role in stabilizing the association of subunits VIa and VIb within the remainder of the bovine heart enzyme.

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 an annulus of membrane phospholipids. The association of cardiolipin (CL)[1] is unique compared with the other mitochondrial membrane phospholipids. In eukaryotes, cardiolipin is present only within the mitochondrial inner membrane where it is tightly associated and functionally important to a number of the dimeric mitochondrial enzymes and translocases, including cytochrome *c* oxidase (*1*−*3*), cytochrome bc_1 (*4*−*9*), glycerol-3-phosphate dehydrogenase (*10*), NADH dehydrogenase *(6, 11*) and the ADP/ATP carrier (*12*, *13*).

The specific requirement of bovine cytochrome *c* oxidase for cardiolipin has been controversial despite a number of studies demonstrating its high affinity binding and functional importance. We have demonstrated that two tightly bound cardiolipins can be extracted from the purified, detergent-solubilized enzyme with large excesses of nondenaturing detergents (*14*). The resulting CL-free enzyme has decreased electron transport activity that is restored only by exogenous CL (*1*, *15*). Furthermore, the resulting CL-free enzyme specifically binds two CL's with high affinity (*2*). An inherent difficulty with this approach has been a partial irreversible loss in activity and a partial loss of some nuclearly encoded subunits caused by the exposure to high concentrations of detergent (*16*). In contrast to our results and those of others (*3, 6, 11*), which clearly demonstrate a function requirement for CL, some experimental approaches have not exhibited strong specificity, e.g., (1) spin-labeled CL has only modest preferential affinity for CL-depleted enzyme produced by repeated ammonium sulfate precipitation in the presence of PC (*17*−*19*); (2) some preparations of CL-free enzyme have not had a unique functional requirement for CL (*20*−*22*); (3) CL has not been detected in the crystal structure of the enzyme (*23*) as would be expected if it were specifically bound to the complex.

We, therefore, have attempted to develop a less perturbing method for removal of CL from cytochrome *c* oxidase that would avoid the problems associated with its extraction by detergent. Enzymatic hydrolysis of cardiolipin by phospholipase A_2 would appear to be an attractive alternative since it is the only approach that completely removes CL from bovine cytochrome bc_1 (*4, 5, 9*). Our recent success in using *Crotalus atrox* PLA_2 digestion to reversibly inactivate cytochrome bc_1 (*9*) and our recently developed, sensitive method for quantitation of CL (*24*), encouraged us to apply the *C. atrox* PLA_2 delipidation procedure to remove CL from cytochrome *c* oxidase. Using this approach, we are now able to demonstrate that CL not only is functionally important to the electron transport activity of bovine cytochrome *c* oxidase, but also that it is essential for maintaining the structural integrity of the complex.

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[1] Abbreviations: PLA_2, phospholipase A_2; CL, cardiolipin or 1,2-diphosphatidyl-sn-glycerol; PC, egg yolk phosphatidylcholine; PE, phosphatidylethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; MOPS, 3-[*N*-morpholino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FPLC, fast performance liquid chromatography; HPLC, high performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Materials. Bovine cytochrome *c* oxidase was prepared from Keilin−Hartree heart particles by the method of Fowler et al. (*25*) with modifications described by Mahapatro and Robinson (*26*). Heme content (9.4−9.9 nmol/mg) and phosphorus content (12−20 mol/mol oxidase) were determined as described previously. Individual drops of purified enzyme (20−25 mg/mL protein, 10 mg/mL sodium cholate, 100 mM phosphate buffer, pH 7.4) were quickly frozen by pipetting the solution into liquid nitrogen. Individual frozen aliquots of enzyme, ~25 uL, were stored at −80 °C. Sodium cholate in the enzyme preparation was exchanged for Triton X-100 or dodecylmaltoside by diluting the enzyme to 1 mg/mL with 1 mg/mL detergent followed by dialysis to remove sodium cholate. The resulting Triton X-100, or dodecylmaltoside-solubilized enzyme was monomeric as judged by sedimentation velocity analysis (*27*, *28*). Cytochrome *c* oxidase concentrations were calculated on the basis of $\epsilon_{422} = 1.54 \times 10^5\ M^{-1}cm^{-1}$ (*29*). Horse heart cytochrome *c* (Type III) was obtained from Sigma Chemical Co. Reduced cytochrome *c* was freshly prepared by dithionite reduction, and excess dithionite was removed by G-25 Sephadex gel filtration. Initial concentrations of ferrocytochrome *c* were determined using $\epsilon_{550} = 29.5\ mM^{-1}cm^{-1}$. Phospholipase A_2 was isolated from *C. atrox* venom as described by Walls and Hanahan (*30*). Its purity was verified by SDS electrophoresis. Only one Coomassie blue stained band at about 15 kDa was observed. Concentrations of phospholipase A_2 were determined using $\epsilon_{280}^{1\%} = 22.7\ M^{-1}cm^{-1}$ (*30*).

The silicic acid HPLC column (5μ Radial Pak Resolve Silica cartridge, 0.8 cm × 10 cm) was purchased from Waters Corporation, Inc.; the C_{18} reversed phase column (10 μ, 4.6 mm × 250 mm, cat. no. 218YP104) was from Vydac; and the HiTrapQ FPLC column was from Pharmacia. Cyclohexane, 2-propanol, and phosphoric acid were of HPLC grade and were obtained from Fisher Scientific. HPLC grade chloroform and methanol were from EM Science. Beef heart cardiolipin and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids; egg yolk L−α-phosphatidylcholine (PC) was purchased from Sigma Chemicals. Ultrapure Triton X-100 and dodecylmaltoside were obtained from Boehringer Mannheim and Anatrace, Inc., respectively. All other chemicals were reagent grade.

Methods. *Phospholipase A_2 Digestion of Phospholipids Bound to Cytochrome c Oxidase*. Cytochrome *c* oxidase (5 μM) was delipidated by PLA_2 (5 μM) at room temperature in 20 mM MOPS, pH 7.2 containing 20% glycerol, 10 mM $CaCl_2$, and 1 mg detergent (dodecylmaltoside or Triton X-100) per mg cytochrome *c* oxidase. The decrease of cytochrome *c* oxidase molecular activity as a function of PLA_2 hydrolysis of CL was determined after quenching the reaction with 50 mM EDTA.

Determination of Cytochrome c Oxidase Activity. Cytochrome *c* oxidase activity was measured spectrophotometrically to an accuracy of ± 5% by following the pseudo-first-order rate of oxidation of 25−30 μM ferrocytochrome *c* by 1.75 nM cytochrome *c* oxidase at pH 7.0 in 25 mM phosphate buffer containing 1 mg/mL dodecylmaltoside (*31*). The activity of purified cytochrome *c* oxidase was 310−350 s^{-1}. The effect of different phospholipids on restoring activity to CL-depleted oxidase was determined by incubation of 0.25 μM cytochrome *c* oxidase at 4 °C with 20 μM CL, PC or DOPE dissolved in 40 mM MOPS buffer, pH 7.2, containing 20% glycerol, 1 mM EDTA, and 1 mg/mL Triton X-100 followed by 150-fold dilution into assay buffer containing 1 mg/mL dodecylmaltoside (*31*).

HiTrapQ FPLC Ion Exchange Chromatography. Anion-exchange column chromatography of cytochrome *c* oxidase on the HiTrapQ column was a modification of the method previously developed for a MonoQ FPLC column (*16*). Chromatography was accomplished using a Laboratory Data Control HPLC system equipped with a Rheodyne manual injector (1-ml loop) and a Gilson variable wavelength detector. Digital absorbance data from the detector was collected using a Waters SATIN A/D interface module connected to a 50 MHz 486 PC computer running Waters Millennium 2010 software version 2.0. Elution buffers were as follows: buffer A was 20 mM MOPS, pH 7.2, that contained 0.5 mg/mL dodecyl maltoside; buffer B was the same as buffer A, but also contained 0.4 M Na_2SO_4. The elution gradient at 0.5 mL/min was: (1) linear gradient from 0 to 25% buffer B in 4 min; (2) isocratic elution with 25% buffer B for 20 min; (3) a linear gradient from 25 to 100% buffer B in 10 min; (4) isocratic elution with 100% buffer B for 10 min; (5) linear reset to 100% buffer A in 12min; (6) reequilibration of the column for 30 min with 100% solvent buffer A to prepare it for the next injection.

Quantitation of Cardiolipin. The CL content of cytochrome *c* oxidase before and after incubation with PLA_2 was quantified by silicic acid HPLC after extraction of phospholipids from partially denatured enzyme (contained 2 M guanidinium chloride) as described previously (*24*). Digital elution data were collected from a Waters PDA detector using the Waters software described above. Assessed errors of the cardiolipin determination were about ± 0.15 nmol. The phospholipid content of PLA_2 treated cytochrome *c* oxidase was also qualitatively analyzed by thin-layer chromatography using Whatman K5 silica gel, 80 Å, plates developed with $CHCl_3$:CH_3OH:H_2O:NH_4OH 65:35:4:0.25, v/v/v/v. Spots were visualized after charring with 6 N H_2SO_4.

Analysis of Subunits. Quantitative determination of the 10 nuclearly encoded subunits (subunits IV−VIII, nomenclature according to Kadenbach et al. (*32*)) was determined by C_{18} reversed phase HPLC (*16*). Presence of the three other subunits, (subunits I−III), was determined by SDS-polyacrylamide gel electrophoresis on 15% acrylamide gels that contained 2 M urea in addition to 0.1% SDS (*14*).

Removal of Triton X-100. Triton X-100 interferes with the quantitation of CL by silicic acid HPLC (*24*); therefore, if Triton X-100 was used during the PLA_2 digestion of CL, it was removed from cytochrome *c* oxidase by exchange for dodecylmaltoside using HiTrapQ ion exchange chromatography prior to analysis of CL. As much as 10 mg of cytochrome *c* oxidase could be applied to a HiTrapQ column that had been equilibrated with buffer A. After washing with buffer A for 15 min at 0.5 mL/min, Triton X-100 free cytochrome *c* oxidase was eluted with a linear gradient from 0 to 100% buffer B in 5 min. Triton X-100 was also quantitatively removed from cytochrome *c* oxidase by incubation with 100 mg SM-2 Bio Beads per mg protein at 4 °C for 2 h (*33*) followed by the addition of 1 mg dodecylmaltoside per mg protein to prevent precipitation.

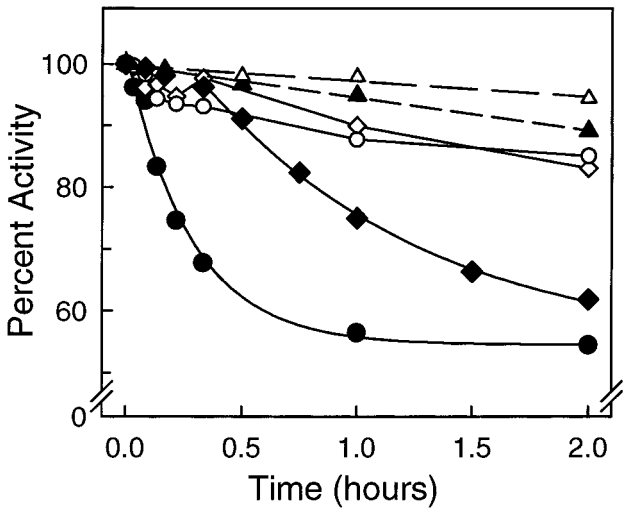


FIGURE 1: Dependence of cytochrome *c* oxidase activity on time of incubation with phospholipase A_2. In each experiment, cytochrome *c* oxidase (5 μM) was incubated at room temperature with PLA_2 (5 μM) in 20 mM MOPS buffer, pH 7.2, containing 20% glycerol with the following additions: (1) Triton X-100 (1 mg/mg protein) and 10 mM $CaCl_2$ (circles); (2) Triton X-100 (1 mg/mg protein) and 1 mM EDTA (triangles); (3) Dodecylmaltoside (1 mg/mg protein) and 10 mM $CaCl_2$ (diamonds). In each case, molecular activity was measured spectrophotometrically after dilution into 1 mg/mL dodecylmaltoside either directly (filled symbols) or after addition of EDTA and exogenous CL (open symbols) (refer to Methods for details). The lines through the data obtained with active PLA_2, i.e., filled circles and diamonds were obtained by nonlinear least-squares fitting of the data to a first-order exponential decay in activity, i.e., $A_t = (A_o - A_\infty)e^{-k(t-t_o)} + A_\infty$; where A_o = initial activity, A_t = activity at time t, A_∞ = activity at infinite time, k = first-order rate constant, and t_o = lag time before exponential decay begins. The best fit parameters for PLA_2 hydrolysis in Triton X-100 were: $A_o = 91.8\%$, $A_\infty = 54.5\%$, $k = 1.00 \times 10^{-3}$ sec^{-1}, and $t_o = 0.07$ s. Best fit parameters for PLA_2 hydrolysis in dodecyl maltoside were: $A_o = 93.4\%$, $A_\infty = 53.4\%$, $k = 0.28 \times 10^{-3}$ sec^{-1}, and $t_o = 0.42$ s. The slower rate of CL hydrolysis in the presence of dodecylmaltoside is consistent with the known activity of PLA_2 in both detergents (*34*).

No significant adsorption of CL or cytochrome *c* oxidase was observed on Bio Beads SM-2 using these conditions. HiTrapQ ion exchange chromatography removed almost all of the Triton X-100, but traces of Triton X-100 were still detected during silicic acid HPLC quantitation of CL after the SM-2 Bio Beads procedure.

RESULTS

Phospholipase A_2 Digestion of Cardiolipin Decreases the Electron Transport Activity of Cytochrome c Oxidase. Treatment of purified, detergent-solubilized cytochrome *c* oxidase (15−20 mol PL per mol enzyme) with PLA_2 in the presence of $CaCl_2$ decreases its electron transport activity by approximately 50% in 2 h. The inactivation has an initial lag phase followed by a first-order loss of activity (Figure 1). Both the extent of the lag phase and the first-order rate of inactivation are dependent upon the type of detergent used to solubilize cytochrome *c* oxidase. For example, the lag phase is about 3−4 times shorter and the rate of inactivation is 3.6 times more rapid when Triton X-100 is the solubilizing detergent rather than dodecylmaltoside (Figure 1). In either detergent, the final activity (evaluated by nonlinear least-squares fitting of the data to a first order reaction) is identical, 54% of the initial value. The reason for the lag phase is not known, but one possible explanation is that loosely bound phosphatidylcholine, phosphatidylethanolamine and nonessential CL are more accessible to PLA_2 than the two tightly bound CL's. In support of this hypothesis, the length of the lag phase is extended when exogenous CL is added to the incubation mixture, i.e., excess CL, which does not bind tightly to enzyme saturated with CL, must be hydrolyzed before the essential CL is hydrolyzed (data not shown).

Table 1: Restoration of Electron Transport Activity to PLA_2-Treated Cytochrome *c* Oxidase by Various Phospholipids

phospholipid	percent enzyme activity compared to nondelipidated cytochrome *c* oxidase[a]
None	55 ± 3
CL	95 ± 5
DOPE	67 ± 2
PC	57 ± 3

[a] Electron transport activity was measured in the presence of 1 mg/mL dodecylmaltoside as described in Experimental Procedures. Activity of nondelipidated cytochrome *c* oxidase was 310−350 s^{-1}. Results are similar to data previously obtained with CL-depleted enzyme prepared by Triton X-100 extraction (*15*).

Complete enzymatic cleavage of the tightly bound CL during the PLA_2-induced loss of cytochrome *c* oxidase activity was confirmed by quantifying the CL that remained after 2 h incubation with PLA_2 in the presence of Triton X-100. Before incubation with PLA_2, purified, fully active cytochrome *c* oxidase contained 3.6 CL per monomer (quantified by silicic acid HPLC analysis). After hydrolysis with PLA_2, the enzyme contained less than 0.05 mol CL per monomer. The absence of CL, PC, and PE was also confirmed by TLC plate analysis. However, the complex did contain 2.9 g-atom organic P per cytochrome aa_3 unit. The source of the additional organic P is not known, but it could be due to either lysophospholipids, or P-containing contaminates, e.g., detergents, buffers, etc. Similar amounts of organic P have been present in our delipidated preparations prepared by detergent extraction of CL, although they too have been completely free of CL, PC, or PE (*2*).

The loss of electron transport activity by exposure to PLA_2 is almost certainly due to the hydrolysis of CL. First, inhibition of phospholipase activity by chelation of Ca^{2+} with EDTA prevents the loss of activity (Figure 1, dashed lines). Second, the inhibition caused by PLA_2 is reversed by exogenous CL (Figure 1), but not by PC or DOPE (Table 1), a result that is nearly identical to that obtained when CL was removed by Triton X-100 extraction (*15*). Third, the cardiolipin content of cytochrome *c* oxidase, during digestion of phospholipids by PLA_2, decreases exponentially at a rate similar to the exponential loss of activity (Figure 2); in fact the decrease in CL content correlates almost perfectly with the decrease of electron transport activity (Figure 2, inset). Last, reassociation of phospholipids with the vacant high-affinity CL binding sites is specific for CL. For example, the CL content increased from less than 0.1 CL to 2.6 ± 0.5 CL per monomer after CL-free cytochrome *c* oxidase was: (1) incubated with a 25-fold molar excess of CL dissolved in 20 mg/mL cholate and 1 mg/mL dodecylmaltoside overnight at 4 °C; (2) dialyzed overnight to remove cholate; (3) separated from excess CL by HiTrapQ ion exchange chromatography. Only 0.5 ± 0.5 mol PC reassociated per cytochrome aa_3 in similar experiments with phosphatidylcholine.

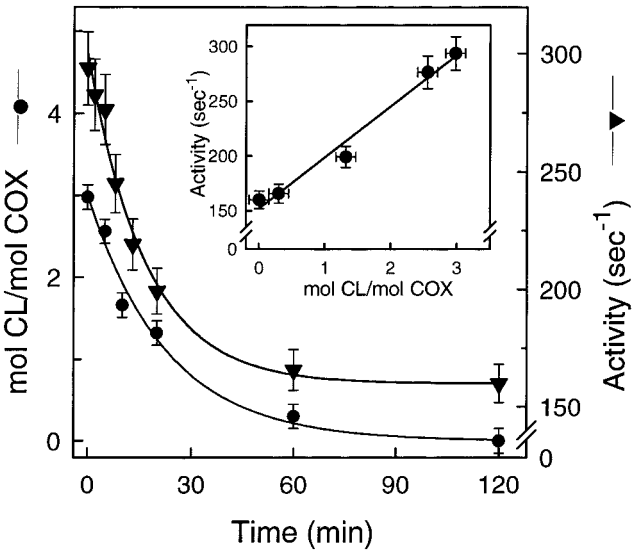


FIGURE 2: Time dependence of cytochrome *c* oxidase molecular activity and CL content as a function of PLA_2 digestion in Triton X-100 and 10 mM $CaCl_2$. Molecular activity was determined spectrophotometrically at pH 7.0 in the presence of 1 mg/mL dodecylmaltoside (filled triangles). CL content per cytochrome aa_3 monomer was quantified by silicic acid HPLC after organic extraction of phospholipids (filled circles). Conditions for PLA_2 digestion, determination of molecular activity, and quantitation of CL content are given in Methods. The error in determination of molecular activity was estimated to be ± 5%; the error in CL determination was estimated to be ± 0.15 nmol. Inset: Correlation between molecular activity and CL content of cytochrome *c* oxidase per cytochrome aa_3.

Subunits VIa and VIb Dissociate When Cardiolipin is Cleaved by PLA_2. Chromatographic elution of purified cytochrome *c* oxidase from a HiTrapQ FPLC ion exchange column separates the intact complex from a form that is missing subunits VIa and VIb (Figure 3). At pH 7.2, 80−85% of the enzyme elutes as the 13-subunit complex (peak A), while the remaining 15−20% elutes as the 11-subunit complex (peak B) (Figure 3, thin line). However, after incubation of Triton X-100 solubilized cytochrome *c* oxidase with active PLA_2 for 2 h, all the enzyme elutes as Peak B (Figure 3, thick line). The conversion to peak B occurs more slowly with dodecylmaltoside solubilized cytochrome *c* oxidase, e.g., approximately 80% of the complex elutes as the 11-subunit complex. This result is consistent with the slower hydrolysis of CL by PLA_2 in dodecylmaltoside [ref *34* and Figure 1]. Subunit analysis of the resulting Peak B by reversed phase C_{18} HPLC chromatography (Figure 4) and gel electrophoresis (not shown) confirms that peak B contains stoichiometric amounts of 11 subunits and is completely devoid of subunits VIa and VIb.

Loss of Subunits VIa and VIb Correlates with Decreased Cardiolipin Content. Conversion of the 13-subunit complex into the 11-subunit complex was quantified as a function of incubation time with PLA_2 and compared with the amount of CL that remains. An almost perfect correlation exists between the percentage of 13-subunit enzyme and the percentage of bound CL (Figure 3, inset). In fact, if 50% of the CL is hydrolyzed, which produces 50% each of peak A and B, the CL content of peak A is more than 2 mol CL per cytochrome aa_3, while the CL content of peak B is less than 0.5 mol CL per cytochrome aa_3. Although removal of CL induces dissociation of subunits VIa and VIb, the reverse process does not occur, i.e., removal of subunits VIa and VIb by exposure to large amounts of detergent or urea does not cause a corresponding decrease in associated CL. For example, the 11-subunit form of the enzyme increases in a time dependent manner as the elution from the HiTrapQ ion

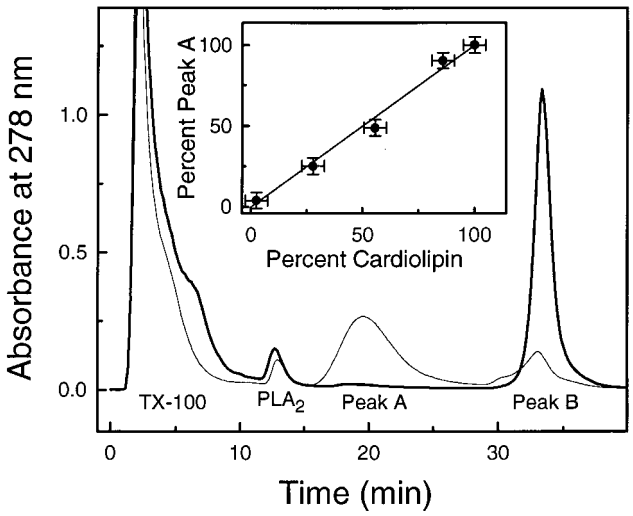


FIGURE 3: Quantitation of 13-subunit and 11-subunit forms of cytochrome *c* oxidase by HiTrapQ anion-exchange chromatography as a function of PLA_2 digestion. Elution of samples incubated with active PLA_2 in the presence of 10 mM $CaCl_2$, or inactive PLA_2 in the presence of 1 mM EDTA are shown by thick and thin lines, respectively. Digestion conditions were as follows: cytochrome *c* oxidase (2.5 nmol) and PLA_2 (2.5 nmol) were incubated at room temperature in 20 mM MOPS buffer, pH 7.2, containing Triton X-100 (1 mg/mL) and either 10 mM $CaCl_2$ or 1 mM EDTA. After 2 h, the reaction was quenched with EDTA and applied to a 1 mL HiTrapQ ion exchange column. Elution conditions and gradients are described in Methods. Inset: Correlation between the percentage of cytochrome *c* oxidase that elutes as peak A (13-subunit form) and the CL content per cytochrome aa_3 monomer after PLA_2 digestion, but before chromatography. Solid line demonstrates ideal 100% correlation between the parameters.

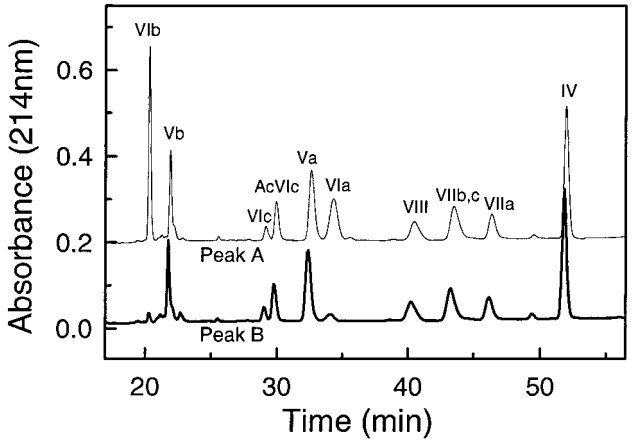


FIGURE 4: Subunit content of peak A and peak B forms of cytochrome *c* oxidase by C_{18} reversed-phase HPLC. Analysis of Peak A form of cytochrome *c* oxidase (upper thin line) was obtained by HiTrapQ chromatography of cytochrome *c* oxidase that was not treated with PLA_2. Analysis of Peak B form of cytochrome *c* oxidase (lower thick line) was obtained by HiTrapQ chromatography of enzyme treated with PLA_2 for 2 h at room temperature in buffer containing Triton X-100. Separation of the 10 nuclearly encoded cytochrome *c* oxidase subunits was performed according to Liu et al. (*16*). Subunits are labeled according to the nomenclature of Kadenbach et al. (*32*). In both cases 100 mg (0.5 nmol) of cytochrome *c* oxidase was analyzed (refer to Methods for details).

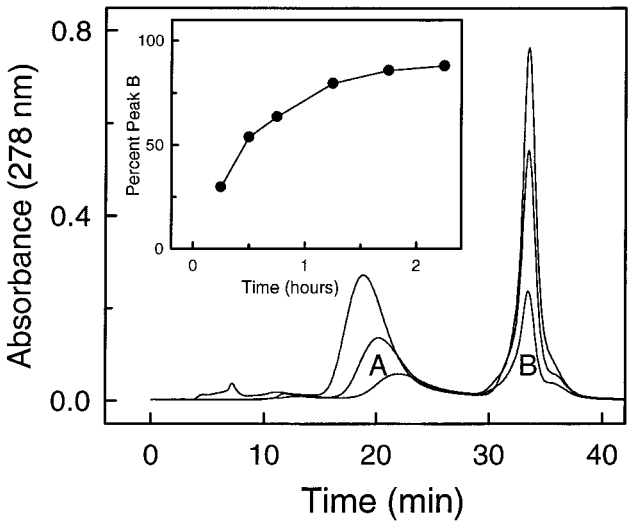


FIGURE 5: Removal of subunits VIa and VIb from cytochrome *c* oxidase by HiTrapQ anion-exchange chromatography. The percentage of Peak B (11-subunit form of cytochrome *c* oxidase) increases as the time before elution gradient begins is delayed. The three chromatographs shown in the main figure correspond to delays in the gradient of 0, 0.5, and 1.5 h. With no delay, cytochrome *c* oxidase elutes in approximately 0.25 h; therefore, each sample was bound to the column for 0.25, 0.75, and 1.75 h, respectively. In each case, 500 mg (2.5 nmol) cytochrome *c* oxidase was loaded and eluted with a Na_2SO_4 gradient as described in Methods. Inset: Percentage of cytochrome *c* oxidase eluting as peak B (11-subunit form) as a function of the total time enzyme was bound to the HiTrapQ column.

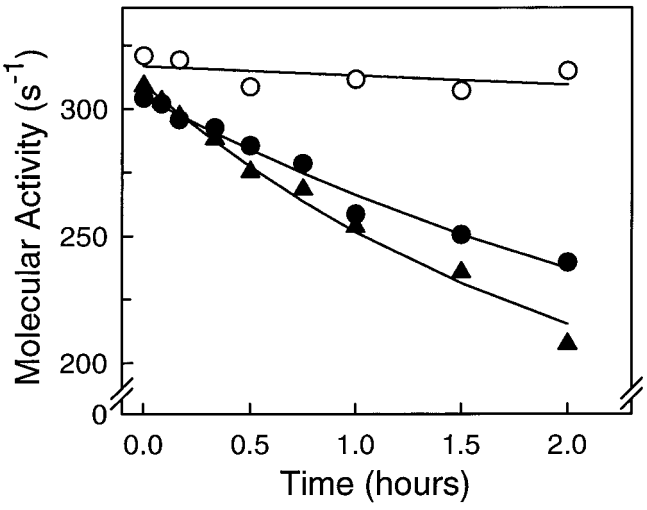


FIGURE 6: Time course for reversible inactivation of the 13-subunit and 11-subunit forms of cytochrome *c* oxidase by PLA_2. Cytochrome *c* oxidase (5 μM) was isolated from the HiTrapQ ion exchange column after 40 min. (refer to Figure 5): 50% as peak A (13-subunit form) and 50% as peak B (11-subunit form). Enzyme in each peak was made 1.0 mg/mL in both dodecylmaltoside and Triton X-100, dialyzed to remove salt, and incubated at room temperature with PLA_2 (5 μM) as described in Methods. Activity of peak A (triangles) and peak B (circles) was measured without (filled symbols) and with (open symbols) addition of exogenous 20 μM CL to 0.25 μM enzyme (refer to Figure 1 and Methods for details). Lines through the activity data before addition of CL are nonlinear least-squares fits of the data to a first-order decay, assuming the final activity was 50% of the initial activity. Best fit values for the first-order rate constants were 1.5 10^{-4} s^{-1} and 0.96 10^{-4} s^{-1} for peak A and peak B, respectively. The peak A form of cytochrome *c* oxidase initially contained 4.0 $\pm$ 0.15 mol CL per aa_3, which decreased to 0.6 after 2 h digestion with PLA_2. The peak B form initially contained 1.8 $\pm$ 0.15 mol CL per aa_3, which decreased to 0.63 after 2 h digestion with PLA_2.

exchange column is delayed (Figure 5). The CL content decreases during this process (1.8−1.9 CL per cytochrome aa_3 for peak B compared with 3.2−4.0 for peak A), but the two CL's that are essential for maximum activity are still present. The molecular activity of both forms is identical (310 s^{-1}), and the activity of neither is increased significantly by exogenous CL (time zero in Figure 6). Furthermore, incubation of either form of the enzyme with active PLA_2 causes a first order decrease in activity that is restored by exogenous CL, i.e., both forms contain the two essential CL's (Figure 6).

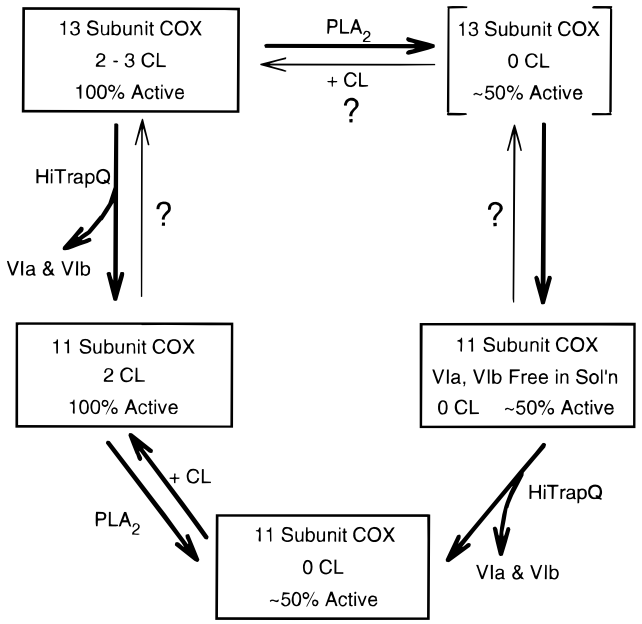
Exogenous Cardiolipin Does Not Induce Reassociation of Subunits VIa and VIb with Cardiolipin-Free 11-Subunit Enzyme. Incubation of the PLA_2-treated enzyme with an 80−100 fold molar excess of CL, for 12−16 h at 4 °C in the presence of 50 mM EDTA, did not alter the elution pattern from the HiTrapQ ion exchange column. If the subunits had reassociated with the 11-subunit enzyme, then an increase in peak A would have occurred. Reassociation could not be detected with either the Triton X-100 or dodecylmaltoside solubilized enzyme at pH's between 7 and 9. Addition of 0−60% glycerol or 10% poly(ethylene glycol) also did not facilitate subunit reassociation. Dissociation of these two subunits appears to be irreversible.

DISCUSSION

Phospholipase A_2 proved to be an effective and specific method for completely removing the tightly bound CL from detergent solubilized bovine cytochrome *c* oxidase. Using this procedure, we have demonstrated that CL bound to cytochrome *c* oxidase is not only functionally important for maintaining full electron transport activity, but also that it stabilizes subunit interactions within the complex. Once CL is removed, the association of subunits VIa and VIb is destabilized and both subunits dissociate from the complex. However, it is the removal of CL, not the dissociation of subunits VIa and VIb that is responsible for the reversible decrease in electron transport activity.

Decreased cytochrome *c* oxidase activity is directly linked to removal of CL and not to subunit loss. A nearly perfect correlation exists between cytochrome *c* oxidase activity and CL content (refer to Figure 2). Full activity is restored to CL-depleted complex in a concentration dependent manner that is highly specific for CL or closely related CL analogues (refs *1* and *2* and Figure 1). Full activity cannot be restored by any process that does not include CL. The loss of subunits VIa and VIb also correlates with CL removal. It might be thought that removal of these two subunits is the primary cause for the altered activity, but this is not the case. Both of these subunits can be removed, without depleting the enzyme of the two functionally important CL's, using a HiTrapQ ion exchange chromatography procedure (refer to Figure 5). Furthermore, the molecular activity of the resulting 11-subunit complex is identical to that of the intact enzyme's and this activity is unaffected by addition of CL. Subsequent PLA_2 hydrolysis of the two CL's that remain bound to the 11-subunit enzyme once again results in a reversible decrease of activity that can be restored by exogenous CL (refer to Figure 6).

Scheme 1. Proposed Mechanism and Functional Consequences of Removing Cardiolipin and Subunits VIa and VIb from Cytochrome *c* Oxidase[a]



[a] Purified, cytochrome *c* oxidase containing all 13 subunits, 2 essential CL, and 1−2 non-essential CL is at the top left-hand corner. We propose that PLA_2 hydrolyzes all of the bound CL and generates an unstable 13-subunit intermediate (top right-hand corner), which has half the enzymatic activity of the initial CL-rich complex. If this intermediate has a relatively short half-life, then it would explain the irreversibility of this step. Subunits VIa and VIb would dissociate immediately, generating the 11-subunit complex (bottom right-hand corner). Subsequent purification of the CL-depleted enzyme by HiTrapQ ion exchange chromatography removes the dissociated subunits and results in the complex shown at the bottom of the figure. Alternatively, subunits VIa and VIb can be removed from the original complex without removal of CL by HiTrapQ column chromatography (left side of Scheme 1). CL can then be removed from the resulting 11-subunit complex by PLA_2, which generates the CL-free, 11-subunit complex (bottom of the figure). In either case, this final product binds exogenous CL, which restores full activity. Steps shown with bold face arrows are part of the present study. Steps shown with thin arrows and question marks are theoretically possible, but may not be technically feasible.

Further evidence that decreased cytochrome *c* oxidase activity is not linked to dissociation of subunits VIa and VIb is the fact that when CL is added to a mixture containing VIa, VIb, and the CL-free 11-subunit complex, the 13-subunit complex does not reassemble. However, full activity is restored even though these two subunits do not reassociate. The irreversibility of subunit removal is somewhat expected since detergent almost certainly coats the apolar surfaces of the separated components which would inhibit reassociation. Nevertheless, activity is restored without reassembly of the 13-subunit complex. This is direct evidence that these activity changes are not a direct consequence of subunit dissociation. A schematic model that summarizes the removal of CL by PLA_2, the dissociation of subunits VIa and VIb together with our interpretation of these data is given in Scheme 1.

The location of the two functionally important CL binding sites within bovine cytochrome *c* oxidase is not known at this time. The CL binding sites are not evident in the three-dimensional crystal structure of Yoshikawa (*23*). Electron density due to CL was not resolved in the electron density map even though chemical analysis indicated that the crystalline preparation contained one CL per cytochrome aa_3 monomer (*35*). Yoshikawa has suggested that CL may be located in the large cavity between the concave surfaces of the two monomeric units that face each other around a quasi-2-fold symmetry axis in the dimeric structure (*35*). If CL is located in this cavity, it could explain the direct linkage we find between the hydrolysis of CL by PLA_2, the dissociation of subunits VIa and VIb from the complex, and the lag phase in the loss of activity (Figure 1). Subunits VIa and VIb are both located near this cavity and thought to stabilize the cytochrome *c* oxidase dimer (*23*). In addition, both VIa and VIb are positively charged at neutral pH, which would favor their interaction with the negatively charged headgroup of CL. However, placing CL in this cleft is inconsistent with chemical labeling studies using arylazido derivatives of CL (*1*, *36*). These studies indicate that one of the smallest subunits, i.e., either VIIa, VIIb, VIIc, or VIII contacts the hydrocarbon fatty acid tails of CL. We have recently used a CL derivative with an arylazido group attached to its polar headgroup (the hydroxyl group on the bridging glycerol) and we have identified labeling of VIIa and VIIc as detected by ESI/MS (Sedlák and Robinson, unpublished). All of these labeling studies suggest that CL is not located in the cavity between the two monomers. Subunits VIIa and VIIc each contain a single membrane spanning α-helix, but they are located on the surface that is distal from the dimer interface (*23*). Clearly, identification of subunits participating in the CL binding site of bovine cytochrome *c* oxidase must await more definitive experimental results.

Last, the present study with PLA_2 agrees with data obtained using CL-depleted enzyme prepared by nonspecific detergent extraction, i.e., extraction of CL with 50 mg per mL Triton X-100 (*1*, *2*, *14*, *31*). However, the PLA_2 delipidation procedure clarifies a complication encountered with detergent extraction of CL. The partial removal of subunits VIa, VIb, and VIIa was thought to be an unwanted complication of detergent extraction (*2*, *16*). In light of the present results, it is evident that dissociation of VIa and VIb is unavoidable since it is directly coupled to CL removal (refer to Figure 4).

One unanswered question remains regarding the functional involvement of CL in maintaining full activity of cytochrome *c* oxidase, i.e., what is the mechanism by which CL removal decreases electron transfer rates between the redox centers? It seems unlikely that CL directly participates in one of the electron transfer reactions. In fact, this possibility can be eliminated since removal of all CL decreases activity by about fifty percent, but does not completely inactivate the enzyme refer to Figure 1 and refs *1* and *2*. A far more likely scenario is that CL binding stabilizes a fully active conformer of cytochrome *c* oxidase. Removal of CL would, therefore, perturb the enzyme so that electron-transfer rates between the redox centers would be decreased. We have not been able to detect any significant perturbations in the visible spectrum of the two heme centers of cytochrome *c* oxidase upon CL removal. However, preliminary studies do indicate that CL removal reversibly decreases the rate of electron transfer from cytochrome *a* to a_3 (J. Ortega-Lopez, L. Sowdal, and N. C. Robinson, unpublished), a finding that is consistent with the altered structural hypothesis.

In summary, we propose that CL bound to bovine cytochrome *c* oxidase serves both a structural and functional

role, but these two processes are directly linked. These roles are very similar to the requirements of bovine cytochrome bc_1 for CL (*9*). In this model, bound CL stabilizes a fully active conformer of cytochrome *c* oxidase in which the association of subunits VIa and VIb is favored. Upon removal of CL, conversion of the enzyme into a less active and less stable conformer is favored, causing the dissociation of subunits VIa and VIb. It is tempting to predict that CL removal and the subsequent dissociation of subunits VIa and VIb induces monomerization of cytochrome *c* oxidase since these two subunits are thought to stabilize the dimer (*23*), but to date we have no direct evidence for this hypothesis.

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