Phospholipase Digestion of Bound Cardiolipin Reversibly Inactivates Bovine Cytochrome bc_1[†]

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ABSTRACT: Phospholipids and tightly bound cardiolipin (CL) can be removed from Tween 20 solubilized bovine cytochrome bc_1 (EC 1.10.2.2) by digestion with *Crotalus atrox* phospholipase A_2. The resulting CL-free enzyme exhibits all the spectral properties of native cytochrome bc_1, but is completely inactive. Full electron transfer activity is restored by exogenous cardiolipin added in the presence of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE), but not by cardiolipin alone or by mixtures of phospholipids lacking cardiolipin. Acidic, nonmitochondrial phospholipids, e.g., monolysocardiolipin or phosphatidylglycerol, partially reactivate CL-free cytochrome bc_1 if they are added together with DOPC and DOPE. Phospholipid removal from the Tween 20 solubilized enzyme, including the tightly bound cardiolipin, does not perturb the environment of either cytochrome b_{562} or b_{566}, nor does it cause the autoreduction of cytochrome c_1. Cardiolipin-free cytochrome bc_1 also binds antimycin and myxothiazol normally with the expected red shifts in b_{562} and b_{566}, respectively. However, the CL-free enzyme is much less stable than the lipid-rich preparation, i.e., (1) many chromatographic methods perturb both cytochrome b_{566} (manifested by a hypsochromic effect, i.e., blue shift of 1.5−1.7 nm) and cytochrome c_1 (evidenced by autoreduction in the absence of reducing agents); (2) affinity chromatographic purification of the enzyme causes pronounced loss of subunits VII and XI (65−80% decrease) and less significant loss of subunits I, IV, V, and X (20−30% decrease); and (3) high detergent-to-protein ratios result in disassembly of the complex. We conclude that the major role of the phospholipids surrounding cytochrome bc_1, especially cardiolipin, is to stabilize the quaternary structure. In addition, bound cardiolipin has an additional functional role in that it is essential for enzyme activity.

Bovine cytochrome bc_1, also known as ubiquinol-cytochrome *c* oxidoreductase or complex III, is a multisubunit integral membrane complex that is part of the mitochondrial electron-transfer chain (*1*, *2*). It catalyzes electron transfer from ubiquinol to cytochrome *c* and couples it to the transfer of protons across the inner mitochondrial membrane (*3*−*5*). The bovine enzyme consists of 11 nonidentical polypeptides (*1*, *2*), three of which contain redox centers (*6*). Two heme B centers are associated with subunit III (cytochromes b_{562} and b_{566}), a heme C is covalently attached to subunit IV (cytochrome c_1), and a [2Fe-2S] center is in subunit V (the Rieske iron−sulfur protein). The functions of the other polypeptides are not fully understood even though complete 3-D-structural information about the complex is now available (*7*−*9*).

The mitochondrial membrane is unique in that it is the only eukaryotic membrane to contain detectable amounts of cardiolipin. It is found predominantly on the matrix side of the inner membrane (*10*) although small amounts may be present in the outer layer of the outer membrane (*11*). The exact function of cardiolipin in mitochondrial membranes is not understood, but hypotheses regarding its role include its involvement in the lateral conduction of protons (*12*−*14*), acting as a proton reservoir at high pH (*15*, *16*), and stabilizing the structural and/or functional integrity of several mitochondrial membrane protein complexes (*12*, *17*).

Cardiolipin is known to be tightly associated with a number of dimeric mitochondrial enzymes and translocases, including cytochrome *c* oxidase (*17*, *18*), cytochrome bc_1 (*1*, *19*−*21*), glycerol-3-phosphate dehydrogenase (*22*), NADH dehydrogenase (*20*), and the ADP/ATP carrier (*23*). Cardiolipin is the only phospholipid that can maintain full electron transport activity of cytochrome *c* oxidase (*17*), and it is required to keep a number of mitochondrial transporters active during purification, i.e., the ADP/ATP carrier; the mono-, di-, and tricarboxylate carriers; the α-ketoglutarate carrier; the aspartate/glutamate and the palmitoylcarnitine and the (acyl)carnitine translocase system (*12*, *24*). It is associated with the F_oF_1-ATPase and is required for its optimal functioning (*25*, *26*). Creatine kinase, a mitochondrial peripheral enzyme, also binds cardiolipin to specific lysine residues (*27*) and this specific interaction may be important for the interaction of creatine kinase with the ADP/ATP translocase.

The structural and/or functional requirement of cytochrome bc_1 for cardiolipin is more equivocal. Detergent solublized, fully active cytochrome bc_1 contains 8−9 molecules of tightly bound cardiolipin (*21*) together with approximately 100−200 molecules of more loosely associated phosphati-

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dylcholine and phosphatidylethanolamine (*28*). Removal of these phospholipids destabilizes the enzyme, but only the early study by Fry and Green (*20*) suggests an absolute requirement of cardiolipin for maximum electron transport activity. It is the purpose of the present study to remove cardiolipin from cytochrome bc_1 in a systematic, nonperturbing fashion so as to clarify its functional and/or structural importance in the complex.

EXPERIMENTAL PROCEDURES

Materials

Cytochrome bc_1 was isolated from frozen bovine heart tissue as previously described (*29*). Tween 20 and dodecyl maltoside were obtained from Boehringer Mannheim and Anatrace, respectively. All phospholipids were purchased from Avanti Polar Lipids. *Crotalus atrox* venom, DEAE Sephacel, TMPD,[1] ascorbic acid, dithionite, antimycin, MTAB, sodium cholate, yeast, and horse heart cytochrome *c* were from Sigma. Myxothiazol was obtained from Fluka Biochemika. Cyclohexane, 2-propanol, and phosphoric acid were each of HPLC grade and obtained from Fisher Scientific. HPLC grade chloroform and methanol were from EM Science. All other chemicals were reagent grade.

Methods

Phospholipid Removal by Phospholipase A_2 Digestion. Phospholipase A_2 (20 mg) was isolated from *Crotalus atrox* venom (700 mg) using the procedure used for the purification of phospholipase A_2 from *Crotalus adamantus* (*30*). The purified enzyme was 99.8% pure by SDS−PAGE or C_{18} reversed-phase HPLC. The phospholipids bound to cytochrome bc_1 (5 μM) were digested with 10 μM of the purified phospholipase A_2 in a pH 7.8 buffer (40 mM Tris-SO_4, 10 mM $CaCl_2$, and 1 mg/mL Tween 20) for 60 min at 25 °C (*31*). PLA_2 cleaves the ester linkage at the sn-2-position in most PLs and both sn-2 positions in CL to generate lysophospholipids and dilyso-CL, which are all easily removed during reisolation of the treated enzyme. The PLA_2-treated complex was, therefore, repurified at 4 °C by affinity chromatography using a 1 mL column of cytochrome *c* covalently attached to Sepharose (*32*) to produce CL-free cytochrome bc_1.

Phospholipid Removal by Detergent Exchange. Cytochrome bc_1 (5 mg/mL = 20 μM) was incubated with Tween 20 (70 mg/mL) in 40 mM Tris-SO_4, pH 7.8 for 10−15 min, diluted 10-fold with the Mono Q buffer A (40 mM Tris-SO_4, pH 7.8, and 0.5 mg/mL Tween 20), filtered through a 0.45 μ Millipore type HV filter, and applied to either a Mono Q or DEAE Sephacel ion exchange column.

Purification of Lipid Depleted Cytochrome bc_1. A series of linear salt gradients of buffer A (defined above) and buffer B (buffer A with 0.8 M Na_2SO_4) were used to elute the detergent treated enzyme from the Mono Q FPLC column (Pharmacia HR 5/5) using a flow rate of 0.5 mL/min: 100−80% buffer A in 4 min; 80−50% buffer A in 30 min; 50−0% buffer A in 6 min; 0% buffer A for 4 min; and reequilibration with 100% buffer A for 30 min. Salt was removed by dialysis and the sample concentrated to about 0.4 mg/mL using a PM 10 Amicon membrane. When a DEAE Sephacel column (0.6 cm × 3 cm) was used, enzyme was applied in buffer A, washed with 5 mL of buffer A containing 0.07 M Na_2S0_4 to remove phospholipase and detergent-phospholipid mixed-micelles and eluted with 5 mL of buffer containing 0.3 M Na_2SO_4.

Quantitation of Phospholipids and Cardiolipin. The phospholipids were extracted from native or delipidated cytochrome bc_1 as previously described (*31*) and quantified by the phospho-molybdate colorometric method after wet ashing in perchloric acid (*33*). Precision of this assay is ±2 nmol. Cardiolipin content was quantified by silicic acid HPLC (*31*).

Cytochrome Spectra. All spectra were recorded digitally using a SLM Aminco 3000 diode array spectrophotometer having 0.36 nm resolution. The cytochrome c_1 difference spectrum (reduced − oxidized) was determined from the ascorbate reduced minus fully oxidized spectrum and quantified using an extinction coefficient of 17.5 mM^{-1} cm^{-1} for $A_{552.5-540nm}$ (ascorbate reduced − fully oxidized) (*34*). The cytochrome b_{562} difference spectrum (reduced − oxidized) was calculated by subtracting the ascorbate-reduced spectrum from the TMPD-ascorbate reduced spectrum. The cytochrome b_{566} difference spectrum (reduced − oxidized) was calculated by subtracting the TMPD-ascorbate reduced spectrum from the dithionite-reduced spectrum. Cytochrome b_{566} derivative spectra were generated from reduced cytochrome b_{566} difference spectra within Microsoft Excel using a "sliding" slope function of nine data points (2.87 nm) centered on each data point. Repeating the process generated second derivative spectra. Total cytochrome *b*, i.e., b_{562} + b_{566}, was quantified using $\epsilon_{562-577nm}$ = 28.5 mM^{-1} cm^{-1} (dithionite reduced − ascorbate reduced) (*35*).

Antimycin and Myxothiazol Binding. Antimycin and myxothiazol were prepared in ethanolic solution (1 mM HCl was included in the case of antimycin) (*36*). Concentration was determined using ϵ_{320nm} = 4.8 mM^{-1} cm^{-1} for antimycin and $\epsilon_{313\ nm}$ = 10.5 mM^{-1} cm^{-1} for myxothiazol. Binding of the inhibitors to delipidated cytochrome bc_1 was determined by adding either 4 μM antimycin or 5 μM myxothiazol to 0.3 μM cytochrome bc_1 and recording the dithionite reduced spectra after 5 min.

Sedimentation Velocity. Analytical ultracentrifuge studies were performed according to Musatov and Robinson (*28*). Cytochrome bc_1 (0.5 mg/mL) was prepared in either a pH 7.8 buffer (40 mM Tris-SO_4) or a pH 7.4 buffer (20 mM Tris-HCl, 1 mM EDTA, and 0.24 M NaCl) at four different concentrations of Tween 20 to produce a final detergent-to-protein ratio of 1.3, 10, 12, and 15 mg/mg. The sedimentation velocity data (20 scans) were collected at 416 nm during centrifugation at 22 000 rpm and 20 °C and the data analyzed as previously described (*28, 37*).

Enzymatic Assays. Enzyme or enzyme-phospholipid mixture was diluted 1000-fold into assay buffer (25 mM Tris-acetate, pH 7.4, 57 mM sodium acetate, and 12 μM ferricytochrome *c*) that was thermostatically controlled at 25 °C. Calcium acetate, 2 mM, was included in all assays involving CL to prevent the binding of cytochrome *c* to the surface of

[1] Abbreviations: BME, β-mercaptoethanol; CL, cardiolipin (diphosphatidylglycerol or di-*O*-(*sn*-3-phosphatdidyl)-*sn*-1′,3′-glycerol); DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; FPLC, fast-protein liquid chromatography; HPLC, high-performance liquid chromatography, MTAB, tetradecyltrimethylammonium bromide; PL, phospholipid; PLA_2, phospholipase A_2; TFA, trifluoroacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

Table 1: Summary of Phospholipid and Cardiolipin Removal from Detergent Solubilized Cytochrome bc_1

chromatographic procedure	detergent	det/pr[a] (mg/mg)	P/bc_1[b]	percent autoreduced cytochrome c_1	percent reducible cytochromes *b*	CL/bc_1[c]
no treatment (stock enzyme)						
none	Triton X-100	0.4−0.6[d]	120−140[d]	0	100	9.2 ± 0.7[e]
detergent exchange						
Mono Q FPLC	dodecyl maltoside	3.0	ND[f]	92	0	ND[f]
Mono Q FPLC	Tween 20	14.0	10 ± 8	92	0	2.7 ± 1.7
DEAE Sephacel	Tween 20	14.0	44 ± 4	25	100[h]	6.5 ± 1.5
DEAE Sephacel	Tween 20	14.0	35 ± 5	25	100[h]	6.5 ± 2.5
	+MTAB[g]	1.4				
phospholipase A_2						
DEAE Sephacel	Tween 20	1.3	13 ± 3	27	100[f]	<0.05
cyt *c* affinity column	Tween 20	1.3	5 ± 2	0	100[f]	<0.05

[a] Ratio of detergent to protein, mg/mg. [b] Nanomoles of inorganic phosphate per nanomole of cytochrome bc_1. [c] Nanomoles of CL per nanomole of cytochrome bc_1. For detergent exchange experiments, CL content was measured by TLC after extraction by $CHCl_3$:MeOH. TLC plates (Whatman K5 silica gel, 80 Å) were developed using the solvent system of Touchstone (*45*). The phosphate content of the CL spot was measured to determine nanomole of CL. For phospholipase A_2 experiments, CL content was measured by silicic acid HPLC (refer to Experimental Procedures). [d] Varies from preparation to preparation. [e] Refer to ref *31*. [f] Not determined. [g] Myristyltrimethylamonium bromide (MTAB), a positively charged detergent, was included to facilitate the extraction of the negatively charged cardiolipin into the detergent micelles, but it did not significantly improve the extraction even though it does facilitate the extraction of cardiolipin from cytochrome *c* oxidase (*46*). [h] λ_{max} of cytochrome b_{566} was blue shifted by 1.5−1.7 nm.

the CL-containing membranes or mixed micelles; otherwise, there is a very significant nonenzymatic reduction of the bound cytochrome *c* by ubiquinol. The enzymatic reaction was initiated by addition of 40 μM decylubiquinol and rate of reduction of cytochrome *c*, using $\Delta\epsilon_{550-540nm}$(reduced − oxidized) = 19 mM^{-1} cm^{-1}, determined from the pseudo-first-order rate constant (*38, 39*). Concentration of decylubiquinol was determined using $\Delta\epsilon_{290nm} = 4.14$ mM^{-1} cm^{-1} (*40*). Concentration of cytochrome bc_1 was determined by using $\Delta\epsilon_{416nm} = 3.02 \times 10^2$ mM^{-1} cm^{-1}.

Reactivation of CL-Free Cytochrome bc_1 by Phospholipids. Cardiolipin-free cytochrome bc_1 (1.2 μM) was incubated for 2 h at 4 °C in 25 mM Tris-acetate, pH 7.4, 57 mM sodium acetate, 15 mM EDTA, 0.20−0.25 mg/mL Tween 20 (carryover from the PLA_2 digestion procedure), 10 mg/mL sodium cholate, and a mixture of DOPC (13 mM), DOPE (13 mM), and CL (0−18 mM). Other anionic phospholipids were investigated similarly by using them instead of CL. In each case, the enzyme was diluted 1000-fold into the Tris-acetate assay buffer defined above.

Subunit Analysis. The subunit composition of native, partially delipidated and CL-free cytochrome bc_1 was analyzed by C_{18} reversed phase HPLC as described previously (*29*) with detection at 214 nm.

RESULTS

Delipidation of Cytochrome bc_1 by Detergent Exchange. Removal of phospholipids and cardiolipin from Tween 20 solubilized cytochrome bc_1 was attempted by a number of methods that have been successfully used for removing phospholipids from other purified membrane proteins. However, none of the attempts to produce CL-free cytochrome bc_1 by exchange for nondenaturing detergents were very successful. Incubation of the enzyme with large excesses of either Tween 20 or dodecyl maltoside, followed by ion-exchange chromatography (DEAE Sephacel or Mono Q FPLC) to remove the resulting phospholipid-detergent mixed micelles, did not produce CL-free cytochrome bc_1. The phospholipids bound to cytochrome bc_1 could be reduced from about 100 to 10−40, but 3−6 molecules of cardiolipin remained bound to cytochrome bc_1 (Table 1). In addition, exposure to high concentrations of detergent followed by purification of the delipidated enzyme by ion-exchange chromatography caused a number of structural perturbations, e.g., autoreduction of cytochrome c_1, nonreducible cytochrome b_{562} and b_{566}, and partial loss of some subunits (Table 1).

Removal of Phospholipids from Cytochrome bc_1 by Phospholipase Digestion. In contrast to the detergent exchange results, phospholipase A_2 digestion was effective in removing all of the bound CL and nearly all of the phospholipids from cytochrome bc_1. The resulting CL-free enzyme could also be repurified by either DEAE Sephacel ion exchange or affinity chromatography without major perturbations in any of the heme centers. After digestion and repurification, the phosphorus content of cytochrome bc_1 was decreased to 5−9 mol of P/mol of cytochrome c_1 of which less than 0.05 mol were CL. Prior to the chromatography step, CL-free cytochrome bc_1 did not exhibit alterations in any of the heme environments (Figure 1). After repurification by affinity chromatography to remove lyso-phospholipids and PLA_2, cytochrome b_{562}, cytochrome b_{566}, and cytochrome c_1 remained fully oxidized and all could be fully reduced by their appropriate reductants (Figure 1, spectra A, B, and C). Repurification by DEAE Sephacel was not quite as successful since partial autoreduction of cytochrome c_1 normally occurred. Both chromatographic methods did cause a minor perturbation of cytochrome b_{566}, resulting in a small blue shift of about 1.5−1.7 nm in λ_{max} of the difference spectrum (inset to Figure 1). These perturbations in cytochrome b_{566} did not occur if the lipid-rich enzyme was similarly chromatographed on either type of resin.

Inhibitor Binding to CL-Free Cytochrome bc_1. The binding of two inhibitors, antimycin and myxothiazol, to cytochrome bc_1 after phospholipase A_2 digestion and repurification by affinity chromatography was identical to the lipid-rich enzyme. Antimycin induced the same red shift in cytochrome b_{562} when it bound to CL-free cytochrome bc_1 as it did when it bound to the native enzyme (Figure 2, top panel). Myxothiazol also bound to CL-free cytochrome bc_1 and

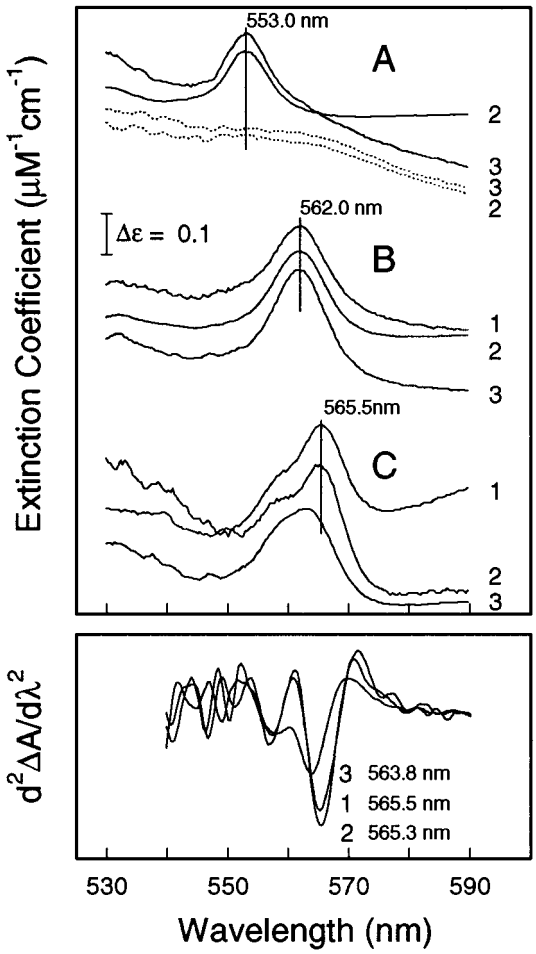

FIGURE 1: Effect of cardiolipin removal and affinity chromatography on the spectral properties of cytochrome bc_1. Spectra were recorded for cytochrome bc_1 solutions at various stages of reduction by ascorbate, TMPD and sodium dithionite. All solutions of cytochrome bc_1 were in 100 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mg/mL Tween 20. In both panels, spectral scans labeled 1, 2, and 3 correspond to (1) lipid rich enzyme as originally isolated, (2) enzyme completely depleted of CL by PLA_2 treatment (prior to affinity chromatography), and (3) enzyme completely depleted of CL by PLA_2 treatment (after purification by affinity chromatography). (Top panel) Spectral scans in group A are those of cytochrome c_1, before (dotted lines) and after (solid lines) reduction by ascorbate. Spectral scans in group B are the difference spectra of reduced minus oxidized cytochrome b_{562}. Spectral scans in group C are the difference spectra of reduced minus oxidized cytochrome b_{566}. Refer to Methods for experimental details on the selective reduction of each cytochrome. (Bottom panel) Second derivatives of the difference spectra recorded for reduced minus oxidized cytochrome b_{566}. All conditions were identical to those in the top panel. Refer to Experimental Procedures for details about taking the second derivatives. The second derivative data clearly indicate that λ_{max} for cytochrome b_{566} is unaffected by removal of CL (spectrum 2) and is blue shifted 1.5−1.7 nm after purification of the CL-free enzyme by affinity chromatography (spectrum 3). The second minimum at 557 nm is nearly identical for all three preparations of enzyme.

induced the typical red shift in the difference spectrum of cytochrome b_{566} (Figure 2, bottom panel) even though the b_{566} reduced minus oxidized difference spectrum was blue shifted by 1.5−1.7 nm (refer to inset to Figure 1). Both of these inhibitor results indicate that the heme B centers were at most minimally perturbed by cardiolipin removal.

Subunit Analysis of CL-Free Cytochrome bc_1. Phospholipase A_2 digestion alone did not significantly alter the

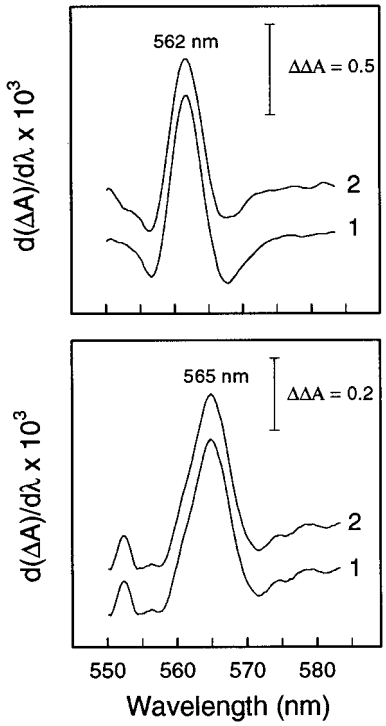

FIGURE 2: Effect of cardiolipin removal upon the antimycin and myxothiazol induced red shifts of the ferrocytochrome *b*s. Spectra are first derivatives of difference spectra produced by the addition of either inhibitor to dithionite reduced enzyme. Detergent and buffer conditions were identical to those described in Figure 1. Spectra labeled 1 are those produced with 0.42 μM lipid-rich enzyme, prior to CL removal by PLA_2 digestion; spectra labeled 2 are those produced with 0.32 μM CL-free enzyme prepared by PLA_2 digestion and purification by affinity chromatography. (Top panel) First derivatives of the antimycin induced difference spectra of reduced cytochrome bc_1. (Bottom panel) First derivatives of the myxothiazol induced difference spectra of reduced cytochrome bc_1.

subunit composition of cytochrome bc_1, but partial depletion of some subunits did occur even after affinity chromatographic repurification, particularly subunits VII and XI (65−80% loss) and to a lesser extent IV, V, and X (Table 2). (Loss of these and other subunits was much greater with all attempts to remove phospholipids by detergent exchange.) The phospholipid environment surrounding the complex appears to be the critical causative factor for subunit loss since it could be eliminated if a mixture of DOPC/DOPE/CL (2:2:1 molar ratio) was added prior to repurification by affinity chromatography.

Self-Association of CL-Free Cytochrome bc_1. Removal of cardiolipin and other phospholipids did not alter the ability of the cytochrome bc_1 complex to dimerize. Both native and CL-free cytochrome bc_1 were homogeneous solutions of dimers, having an $s_{20,w}$ value of about 14 S at low concentrations of Tween 20 (~1.5 mg/mL) (Figure 3, plots a and A). In contrast to the lipid-rich enzyme, however, increasing the concentration of Tween 20 to 7.5 mg/mL caused the CL-free enzyme to dissociate into a distribution of smaller particles (compare plots B and C with plots b and c in Figure 3). Sedimentation coefficients for at least part of this distribution are significantly smaller than would be expected for monomeric enzyme. This, together with extreme heterogeneity of the sample as evident from the nonsymmetrical time derivatives (inset to Figure 3), suggests that high detergent concentrations caused dissociation of subunits from

Table 2: Quantitation of the Subunit Composition of the Different Forms of Phospholipid Depleted Cytochrome bc_1[a]

	no treatment[b]		stoichiometry (mol/mol)		
subunit	peak area (V s/nmol)	stoichiometry (mol/mol)	Tween 20 + Mono Q[c]	PLA_2 + affinity chrom[d]	PLA_2 + PLs + affinity chrom[e]
I	3.76	1.00	0.99	0.81	0.93
II	3.99	1.00	1.11	0.98	0.90
IV	0.3	1.00	1.28	0.78	0.96
V	1.5	1.00	0.31	0.65	0.87
VI	1.56	1.00	0.95	0.86	0.99
VII	1.19	1.00	0.51	0.20	0.80
VIII	0.65	1.00	1.03	0.94	0.98
IX	0.23	1.00	0.92	0.94	1.18
X	0.81	1.00	0.98	0.67	0.92
XI	0.97	1.00	0.10	0.34	0.86

[a] Subunits of cytochrome bc_1 were analyzed by reversed phase C_{18} HPLC as described in Experimental Procedures. Subunit elution was monitored at 214 nm and the area of each peak (V s/nmol) determined by dividing the area (V s) by nanomoles of enzyme injected. The molar ratio of each subunit in native enzyme was assumed to be 1 per complex. The areas for each subunit for the native complex was then used to calculate stoichiometries for each subunit in each form of phospholipid depleted enzyme. Subunit III was not included in the calculations because it is not detected by C_{18} HPLC. [b] Enzyme, as isolated, before any delipidation treatment. [c] Enzyme partially depleted of phospholipids by detergent exchange and repurified by MonoQ FPLC. [d] Enzyme completely depleted of phospholipids, including tightly bound CL, by PLA_2 digestion followed by repurification by affinity chromatography on a cytochrome *c* column. [e] Enzyme completely depleted of phospholipids, including tightly bound CL, by PLA_2 digestion followed by repurification by affinity chromatography after reconstitution with a mixture of PLs (DOPC/DOPE/CL in a molar ration of 2:2:1).

the complex, rather than dissociation of the dimers into monomers.

Activity of CL-Free Cytochrome bc_1. Cardiolipin-free cytochrome bc_1 was totally inactive, but it could be reactivated by certain phospholipids, particularly CL. Ninety percent of the total native activity could be restored to the CL-free enzyme, provided that the complex was not repurified by either cytochrome *c* affinity or DEAE Sephacel chromatography (Figure 4). The reactivation curve produced by CL addition can be fitted to an allosteric type of reaction. The fitting parameters indicate that maximum activation of CL-free cytochrome bc_1 occurs upon the binding of 3−4 molecules of cardiolipin. Restoration of activity was quite specific for cardiolipin although mono-lysocardiolipin and phosphatidylglycerol were able to restore 33 and 28% of the total recoverable activity, respectively (Table 3). Neither phosphatidylserine nor phosphatidic acid, both of which are also negatively charged phospholipids, restored activity, nor could mixtures of phosphatidylcholine or phosphatidylethanolamine.

Attempts to restore activity to CL-free enzyme that had been repurified by either DEAE Sephacel or affinity chromatography were not completely successful. Partial restoration of activity could be obtained if the enzyme was repurified by affinity, but not by DEAE Sephacel chromatography. In the former case, a mixture of CL/DOPC/DOPE (1:2:2 molar ratio) restored about 23% of the total native activity (Figure 5, plot B). Activity was not restored by Tween 20 or sodium cholate solubilized DOPC, CL, DOPC/DOPE or DOPC/CL mixtures. However, addition of DOPC/DOPE/CL (2:2:1 molar ratio) to the CL-free enzyme prior to chromatography on the affinity column resulted in 85% of full activity, i.e., the same conditions that prevented loss of subunits from CL-depleted cytochrome bc_1, also restored and maintained activity during affinity chromatography.

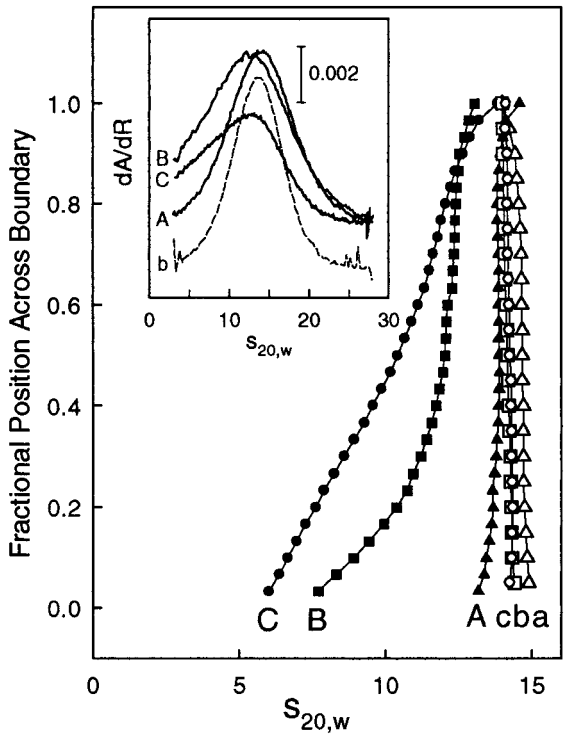

FIGURE 3: Effect of cardiolipin removal upon the homogeneity of Tween 20 solubilized cytochrome bc_1. The homogeneity of cytochrome bc_1, before and after the removal of cardiolipin, was determined by sedimentation velocity centrifugation as a function of the Tween 20 concentration. Sedimentation velocity data were collected at 416 nm during centrifugation at 22 000 rpm and 20 °C. Data were analyzed by both the van Holde−Weischet method (*47*) (main panel) and the time derivative method of Stafford (*48*) (inset panel). (Main panel) Data obtained by the van Holde−Weischet analytical method for 2.2 μM lipid-rich enzyme (open symbols) and for 2.5 μM CL-free enzyme (filled symbols). Data sets a, b, and c were obtained with lipid-rich enzyme containing 1.3 (open triangles), 10.0 (open squares), and 14.8 (open circles) mg of Tween 20/mg of enzyme. Data sets A, B, and C were obtained with CL-depleted enzyme containing 1.6 (filled triangles), 7.8 (filled squares), and 15.8 (filled circles) mg of Tween 20/mg enzyme. In each case 20 scans taken at 8 min intervals were analyzed. (Inset panel) Time-dependent derivative data as generated by the Stafford analytical method for lipid-rich enzyme, before the removal of CL (dashed line) and for PLA_2 treated, affinity column purified enzyme (solid lines). Lines labeled A, B, C, and b correspond to the data with these labels in the main panel. Only one set of data is given for the lipid-rich enzyme since the data are nearly superimposable. The nearly identical values for $s_{20,w}$ across the boundary for the lipid-rich enzyme at all concentrations of Tween 20 and for the CL-free enzyme at a low Tween 20 concentration (1.6 mg/mL) indicate that all of these cytochrome bc_1 preparations were homogeneous and dimeric. The time-derivative data obtained with these preparations, which are symmetrical bell shaped curves centered at 15 S (inset panel), are also consistent with this conclusion. However, higher concentrations of Tween 20 (10.0 and 15.8 mg/mL) induce dissociation of the CL-free enzyme into lower molecular weight species that are not representative of monomeric enzyme.

DISCUSSION

Phospholipase A_2 digestion is the only procedure that removes all the tightly bound cardiolipin from cytochrome

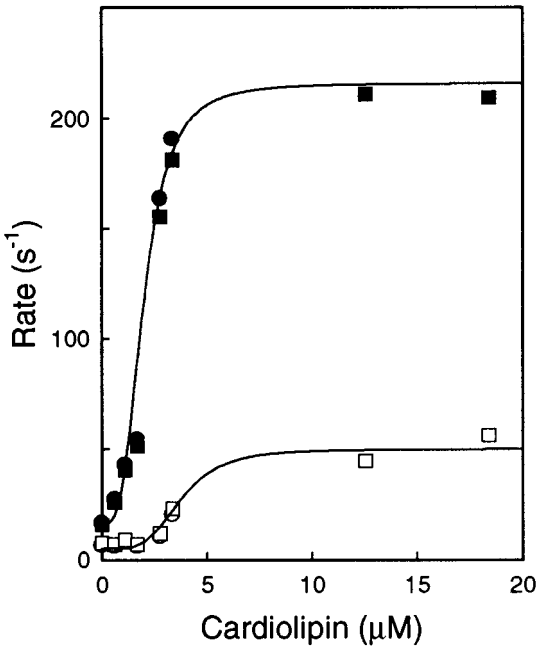

FIGURE 4: Restoration of full electron transport activity to CL-free cytochrome bc_1 is dependent upon cardiolipin concentration. The activity of cardiolipin free enzyme, as a function of CL concentration, was determined before (filled symbols) and after (open symbols) its purification by affinity chromatography. In each experiment, 1.2 μM CL-free cytochrome bc_1 was incubated for 2 h with a mixture of 13 mM DOPC, 13 mM DOPE, and varying concentrations of CL (0−18 mM) in the presence of 1% sodium cholate. With CL-free enzyme that had not been repurified by affinity chromatography (filled symbols), 15 mM EDTA was included in the incubation mixture to inactivate the PLA_2. After the incubation with PLs, the enzyme-phospholipid mixture was diluted 1000-fold into assay buffer and the activity determined from the rate of reduction of 12 μM ferricytochrome *c* by 40 μM decylubiquinol. The solid theoretical lines were obtained by nonlinear least-squares fitting of the data to the allosteric Hill Equation, i.e., Rate $= k_{cat}[CL]^n/(S_{0.5}{}^n + [CL]^n)$ to obtain values for k_{cat} (maximum enzymatic rate), n (Hill coefficient in mole of CL bound per complex); and $S_{0.5}$ (the CL concentration required to reach one-half k_{cat}). The best-fit parameters were (a) $k_{cat} = 213$ s^{-1}, $n = 3.3$, and $S_{0.5} = 2.0$ μM for CL-free cytochrome bc_1 without purification by affinity chromatography; (b) $k_{cat} = 45$ s^{-1}, $n = 4.0$, and $S_{0.5} = 3.8$ μM for CL-free cytochrome bc_1 with purification by affinity chromatography. Activity for the lipid-rich enzyme, prior to removal of CL, under these conditions was 220 s^{-1}.

Table 3: Ability of Different Phospholipids to Reactivate CL-Free Cytochrome bc_1[a]

phospholipid	concentration in assay (μM)	specific activity (s^{-1})[b]
monolysocardiolipin	6	66 ± 3
	15	67 ± 2
phosphatidic acid	6	4 ± 1
	15	4 ± 2
phosphatidylglycerol	6	55 ± 2
	15	55 ± 4
phosphatidylserine	6	5 ± 2
	15	7 ± 2

[a] Cardiolipin-free cytochrome bc_1 (1.2 μM) was incubated with each phospholipid at 4 °C for 1.5 h and then diluted 1000-fold into the assay buffer. [b] Specific activity is in micromoles of cytochrome *c* reduced per second per micromole of cytochrome bc_1. Activity of enzyme, as isolated, before delipidation under these conditions was 220 s^{-1}.

bc_1 without causing irreversible inactivation. The resulting lipid-free complex is devoid of electron-transfer activity, but phospholipid mixtures containing cardiolipin stabilize the enzyme and restore or prevent loss of activity. Others have used phospholipase A_2 digestion to completely delipidate cytochrome bc_1, but, in contrast to our findings, such treatments destabilized the enzyme to such an extent that further studies were precluded (*1*, *19*).

Restoration of Activity to CL-Free Cytochrome bc_1. Phospholipid mixtures containing acidic phospholipids, e.g., CL dissolved in DOPC and DOPE, restore full electron transfer activity to the completely inactive, CL-free enzyme, provided that it is not chromatographically repurified (refer to Figure 4 and Table 3). The role of CL in restoring activity could be either structural or functional. Cardiolipin clearly stabilizes the enzyme and prevents both irreversible inactivation and dissociation of subunits during chromatography. Therefore, CL could be preventing a similar dissociation of subunits and inactivation when the enzyme is diluted into assay buffer, i.e., CL acts only as a stabilization factor. The fact that the CL-free enzyme is completely devoid of activity immediately upon dilution into the assay and that all of the activity is restored by addition of exogenous CL without any apparent kinetic factor, suggests a more functional role for CL. We believe the most plausible explanation is that CL is either essential for catalytic function, or that it acts as an allosteric ligand that stabilizes the fully active conformation. In either case, addition of CL or other negatively charged PLs to CL-free enzyme is required for full enzymatic activity. The most effective mixture is a 1:2:2 molar ratio of CL:DOPC:DOPE, a phospholipid mixture that is similar in composition to the mitochondrial inner membrane. No other lipid mixture is effective, e.g., cardiolipin alone, or mixtures of DOPC and DOPE that do not contain CL. The only exceptions to this are nonphysiological phospholipid mixtures that contain either monolysocardiolipin or phosphatidylglycerol. Mixtures of DOPC and DOPE that contain either of these negatively charged phospholipids restore about one-third of the initial activity (Table 3). The concentration of anionic phospholipid that is dissolved in the DOPC/DOPE mixture correlates with the extent of reactivation in a concentration-dependent manner. This saturation type behavior for restoration of activity is qualitatively similar to the effect of cardiolipin addition to CL-free cytochrome *c* oxidase (*17*) and suggests that binding of cardiolipin to cytochrome bc_1 is a prerequisite for restoring activity. In contrast to cytochrome *c* oxidase, the cardiolipin dependence of cytochrome bc_1 involves a lag phase response, i.e., the activity as a function of the CL concentration is sigmoidal. The reason for the lag phase is not known, but suggests that more than one cardiolipin binds cooperatively. Therefore, we conclude that (1) cardiolipin binding to cytochrome bc_1 is required for full activity, (2) functional binding is specific for anionic phospholipids, and (3) complete restoration of activity is specific for cardiolipin and probably requires the cooperative binding of more than one cardiolipin. These results conflict with earlier studies in which activity could not be restored to lipid-free enzyme (*19*) or partial activity was restored by phosphatidylcholine or phosphatidylethanolamine, but not by cardiolipin (*1*).

Stability of the CL-Free Complex. Cardiolipin-free enzyme, prior to chromatographic repurification, is indistinguishable from the phospholipid-rich, detergent-solubilized enzyme in terms of its visible spectral properties, binding of inhibitors, subunit composition, and self-association into dimers (refer

to Figures 1−4 and Table 2). However, the enzyme is structurally much less stable. Perturbations of the cytochrome b_{566} and c_1 centers and loss of some smaller subunits occurs more easily, particularly if chromatographic purification is attempted with strong ion-exchange resins (refer to Tables 1 and 2). Even using the least perturbing methods, i.e., an affinity column of cytochrome *c* covalently attached to Sepharose, chromatography of the CL-free enzyme causes a small blue shift of 1.7 nm in the cytochrome b_{566} spectrum and partial loss of subunits VII and XI. Stronger chromatographic resins, e.g., DEAE Sephacel and Mono Q, cause partial depletion of several more subunits, including subunits IV, V, VII, X, and XI (refer to Table 2). Full activity also cannot be restored to the purified lipid-depleted enzyme that is structurally perturbed. For example, only a fraction of the total recoverable activity can be restored after repurification by the least perturbing method, i.e., cytochrome *c* affinity chromatography, and no activity can be recovered after reisolation by either DEAE Sephacel or Mono Q FPLC. None of these structural perturbations or irreversible loss of activity is present after the initial digest with phospholipase A_2. Therefore, we conclude that none of these changes occurs coincidently with the removal of phospholipids and CL, but these structural alterations occur later due to instability of the resulting lipid-free complex. This conclusion is somewhat different from that of others in which phospholipid depletion has been suggested as directly causing autoreduction of cytochrome c_1 (*41*), losing the multiplicity of the *b* cytochromes (*42*) and abolishing antimycin binding (*43*).

Role of Phospholipids Bound to Cytochrome bc_1. The hypothesis that a layer of phospholipid must surround cytochrome bc_1 in order to maintain its structural stability is not new for it was proposed by Yu et al. (*41*, *44*) more than 20 years ago to explain their data. All of our data supports their hypothesis. Not only is the lability of the lipid-free complex evident from our chromatographic studies, but in the absence of phospholipids, the enzyme is very sensitive to the concentration of the solubilizing detergent. Increasing the concentration of Tween 20 normally does not affect the hydrodynamic properties of cytochrome bc_1 and it remains dimeric even with 10−20 mg of detergent/mg protein (*29*). However, the CL-free enzyme is more sensitive to high concentrations of Tween 20 and disassembles into smaller particles (refer to Figure 3). Most likely this heterogeneity is caused by dissociation of subunits (refer to Table 2). The subunits that easily dissociate from CL-free cytochrome bc_1, i.e., subunits IV, V, VII, X, and XI, are those that are only anchored to the complex by single transmembrane helical domains and are located at the perimeter of the complex (*7*−*9*). The perturbation in cytochrome b_{566} and the autoreduction of cytochrome c_1 that occur after removal of the boundary layer phospholipid probably result from loss of critical contacts involving these subunits. Therefore, the critical involvement of the phospholipid layer surrounding cytochrome bc_1 could be envisioned as stabilizing the association of subunits IV, V, VII, X, and XI and, thereby, preventing perturbations of the redox centers.

Cardiolipin-Binding Site. Even though the present data support specific and critical structural and functional roles for CL bound to bovine cytochrome bc_1, these studies do not provide any information about the CL-binding location or a direct mechanism by which it could affect function. Studies on creatine kinase indicate that lysine residues are important for cardiolipin binding to that enzyme (*27*). In cytochrome bc_1, subunits IV, V, and VII, which are those that dissociate after removal of CL, are in close contact on the face of the molecule that is distal to the dimer interface (*7*−*9*). Furthermore, near the membrane surface on the matrix side, these subunits contain a cluster of arginine and lysine residues. We speculate that analogous to creatine kinase, cardiolipin may bind near these positively charged residues and stabilize the association of these subunits with the rest of the complex.

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