Investigation of the Essential Boundary Layer Phospholipids of Cytochrome *c* Oxidase Using Triton X-100 Delipidation†

Neal C. Robinson,* Fredlein Strey, and Linda Talbert

ABSTRACT: Beef heart cytochrome *c* oxidase was initially delipidated by incubation of the complex in 5% Triton X-100 followed by separation of the resulting detergent–protein complex from the detergent–lipid mixed micelles by sedimentation through a glycerol gradient containing 1% Triton X-100. After this treatment, the complex contained 2–3 mol of diphosphatidylglycerol (DPG) per heme aa_3. Further delipidation could be achieved by a second 5% Triton X-100 incubation and a second glycerol gradient. After the second Triton X-100 treatment, the complex contained only 1–2 mol of DPG per heme aa_3. The electron transport activity of the resulting delipidated complex was a function of the amount of bound DPG. Complex containing 2–3 molecules of DPG per heme aa_3 was fully active in Tween 80 or Tween 20 while enzyme containing a single molecule of DPG retained only 60% of the original electron transport activity when assayed in either Tween 80 or Tween 20. The original activity of this maximally delipidated complex could be regenerated by its incubation with 1% Triton X-100 containing 0.02% DPG but not by a similar incubation in either phosphatidylcholine or phosphatidylethanolamine. These results are interpreted in terms of three classes of boundary layer phospholipids that have different affinities for the enzyme. The one with the lowest affinity is nonessential for activity and can be replaced by a variety of exogenous phospholipids and detergents. The second class is more tightly bound to the enzyme, requiring high concentrations of Triton X-100 for its removal, and is also nonessential for activity. The third class has the highest affinity for cytochrome *c* oxidase and is comprised of two to three molecules of DPG that are either tightly bound at the enzyme surface or buried in the cytochrome *c* oxidase complex. These DPG molecules are essential for the maximal activity of the complex and cannot be replaced by exogenous phospholipids and detergents, other than DPG, without loss of activity.

Cytochrome *c* oxidase, the terminal electron transport complex of the inner mitochondrial membrane, is a multisubunit enzyme which requires a complete boundary layer of phospholipids for full activity (Jost et al., 1973, 1977). Attempts to remove the boundary layer phospholipids from the enzyme have resulted in the loss of electron transport activity unless exogenous phospholipids were added to the lipid-depleted enzyme (Awasthi et al., 1971; Yu et al., 1975; Brierley & Merola, 1962). Previously we have shown that three-fourths of the boundary layer lipid of cytochrome *c* oxidase can easily exchange with nondenaturing detergents without loss of electron transport activity when assayed in the presence of detergents containing long chain unsaturated or short chain saturated fatty acids (Robinson & Capaldi, 1977). This partially delipidated complex has been used by Vik & Capaldi (1977) for a similar study in which the bound detergents were replaced by exogenous phospholipids. In agreement with the detergent results, the fatty acid composition, but not the polar head group of the added phospholipid, influenced the activity of the complex. The remaining one-fourth of the boundary layer lipids (12–16 mol of P per mol of enzyme) which do not readily exchange with nondenaturing detergents are predominately diphosphatidylglycerol (DPG)[1] (Robinson & Capaldi, 1977). At present, it is not known whether these lipids do not exchange with detergents because they are buried within the complex, because they are tightly bound at the surface of the enzyme, or because DPG does not dissolve well in detergent micelles. It is also not clear if DPG must occupy these sites to achieve full activity since DPG did not exchange with detergents (Robinson & Capaldi, 1977) and attempts to exchange the DPG with exogenous phospholipids have not been successful (Vik & Capaldi, 1977).

In order to investigate the functional importance of the nonexchangeable phospholipids of cytochrome *c* oxidase, we have continued our efforts to develop mild delipidation procedures, using nondenaturing detergents, while maintaining a soluble complex with full activity. Here we report a method for the removal of 85% of the tightly bound phospholipids by using high concentrations of Triton X-100 in combination with glycerol gradient sedimentation. Our results indicate that cytochrome *c* oxidase containing a single mole of diphosphatidylglycerol per mole of complex retains the potential for full electron transport activity. We have, therefore, been able to assess the minimum phospholipid requirements of the complex.

Experimental Procedure

Materials

Cytochrome *c* oxidase was isolated by the method of Fowler et al. (1962) as modified by Capaldi & Hayashi (1972) from beef heart mitochondria prepared according to Crane et al. (1956). Typical preparations had a molecular activity of 150–165 μmol of cytochrome *c* oxidized per s per μmol of heme aa_3 when assayed in 0.5% Tween 80 and contained 8.6–9.4 nmol of heme *a* per mg of protein. Type III cytochrome *c*,

† From the Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284. *Received December 19, 1979.* This investigation was supported by a research grant from the National Institutes of Health (GM 24795) and by an Institutional Research Grant from the University of Texas Health Science Center at San Antonio (GRS-06S000172-11). A preliminary report of some of this work was presented at the 63rd Meeting of the American Society of Biological Chemists (Robinson, 1979).

[1] Abbreviations used: TX, Triton X-100; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PL, phospholipid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Table I: Extraction of Phospholipids from Cytochrome *c* Oxidase[a]

aqueous sample composition	% extraction of phospholipids[b]	
	delipidated cytochrome *c* oxidase[c]	initial prepn of cytochrome *c* oxidase[d]
0.02 M Tris, 0.09 M NaCl (pH 8.1)	92	93
1 M NH_4OH	89	93
1 M HCl	103	96
1 M acetic acid	73	

[a] Extraction was done by using the method of Bligh & Dyer (1959). [b] Percent extraction is based upon the amount of P in the $CHCl_3$ layer compared with the amount of P found in the initial sample prior to extraction. [c] Contained 3.7 mol of P per mol of heme aa_3 complex prior to extraction. [d] Contained 28 mol of P per mol of heme aa_3 complex prior to extraction.

Triton X-100 (TX), Tween 80, Tween 20, bovine heart diphosphatidylglycerol (DPG), and egg phosphatidylcholine (PC) were purchased from Sigma Chemical Co. All other chemicals were ACS certified reagent grade and purchased from Fisher Chemicals.

Methods

Assay Procedures. Cytochrome *c* oxidase concentrations were measured by the biuret method using serum albumin as a standard (Gornall et al., 1949). The heme *a* content of the enzyme was calculated by using an extinction coefficient of 16.5 mM^{-1} cm^{-1} for the difference in absorbance ($A_{605} - A_{630}$) of the dithionite-reduced enzyme (Yonetani, 1961; Griffiths & Wharton, 1961). For routine analysis of dilute samples obtained during the delipidation procedure, the heme aa_3 concentration was based upon $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1} (van Gelder, 1978), a value that was verified for our cytochrome *c* oxidase preparations based upon the heme *a* content.

Cytochrome *c* oxidase activity was measured spectrophotometrically by following the oxidation of reduced cytochrome *c* at 550 nm in either 0.5% Tween 80 or Tween 20 in a 0.01 M phosphate buffer, pH 7.0, at 25.0 °C with a PM 6K Zeiss spectrophotometer equipped with a temperature-controlled cuvette holder. Molecular activities were calculated from the first-order rate constant of the reaction using 25–30 μM cytochrome *c* and the heme aa_3 concentration as described by Vanneste et al. (1974). Activities of the delipidated enzyme in Tween 80 are expressed as a percent of the initial activity of the nondelipidated enzyme in Tween 80. Similarly, the activities of the enzyme in Tween 20 are expressed as a percent of the activity of the nondelipidated enzyme in Tween 20. Stimulation of the molecular activity by DPG, PC, or PE was measured by incubation of the enzyme (0.05 mg/mL) with 0.2 mg/mL lipid in 1% TX and 0.02 M Tris buffer at pH 8.1 containing 0.09 M NaCl and 0.1 mM EDTA for 10–30 min at 0 °C, followed by a 150-fold dilution into an assay cuvette containing 0.01 M phosphate buffer at pH 7.0 and either 0.5% Tween 80 or 0.5% Tween 20.

Phospholipid Analysis. Phospholipids were extracted from cytochrome *c* oxidase at pH 8.1 by using the method of Bligh & Dyer (1959). It was not necessary to extract the phospholipids from a basic NH_4OH solution as described by Awasthi et al. (1971) since similar yields (95%) were obtained when phospholipids were extracted from either the original cytochrome *c* oxidase preparation or the delipidated enzyme in either pH 8.1 buffer, 1 M NH_4OH or 1 M HCl (Table I). Total phospholipid was measured by using the procedure of Chen et al. (1956) after digestion of either the entire protein–lipid complex or the extracted phospholipids in 0.5 mL of perchloric acid according to Marinetti (1962). The phosphorus content of lipids that had been separated by thin-layer chromatography was determined by scraping uniform areas of the silica gel (1.3 × 2.0 cm) into the digestion tubes. The presence of silica gel during the perchloric acid digestion and subsequent color development did not affect the assay of phospholipid standards.

The phospholipids extracted from cytochrome *c* oxidase were separated according to head groups by using thin-layer chromatography on silica gel G plates obtained from Quantum Industries. Mitochondrial lipids containing lysophosphatidylcholine and lysophosphatidylethanolamine were used as standards. (The lysophospholipids in the standard were generated from their corresponding plasmalogens by cleavage of the vinyl ether bond by HCl and $HgCl_2$.) Lipids were visualized prior to P analysis by charring the plate with 6 N H_2SO_4. Large amounts of TX were present in the extracted phospholipids (10–20 mg of TX per 50 nmol of PL) and interfered with the P analysis and thin-layer chromatography; therefore, TX was removed from the spotted sample by developing the plate several times in the first dimension with acetone. The phospholipids which remained at the origin after the acetone development were separated in the second dimension by using chloroform–methanol–water–concentrated NH_4OH (65:35:4:0.25 by volume) as the solvent.

The fatty acid composition of the phospholipids was analyzed by gas–liquid chromatography after conversion to their methyl esters by refluxing in 3% methanolic HCl at 95 °C for 3 h. The methyl esters were extracted with petroleum ether and dried with Na_2SO_4 and $NaHCO_3$. The samples to be analyzed were scraped from the thin-layer plates after visualization with the fluorescent spray toluidinylnaphthalenesulfonate. Neither the fluorescent spray nor the silica gel interfered with the analysis of phospholipid standards.

Polyacrylamide Gel Electrophoresis. Prior to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, protein samples were denatured by heating at 37 °C for 1 h in 2% sodium dodecyl sulfate and 10 mM dithiothreitol and dialyzed against the appropriate loading buffer. Samples containing 20 μg of protein were analyzed by using either (1) 12.5% acrylamide gels in 0.1% sodium dodecyl sulfate and 8 M urea according to Swank & Munkres (1971) or (2) a stacking gel system (4.5% acrylamide in the stacking gel and 15% acrylamide in the running gel) in 0.1% sodium dodecyl sulfate according to Maizel (1971) with the exception that 2 M urea was included in the running gel to give better separation of the smaller molecular weight subunits. Polyacrylamide gels were stained for protein and destained according to Fairbanks et al. (1971) after the urea was removed by soaking the gel in methanol–water–acetic acid (50:40:10 by volume). After being destained, the gels were scanned at 590 nm with a Helena Laboratories Quick Scan R and D densitometer.

Glycerol Gradient Sedimentation in 1% TX. Cytochrome *c* oxidase and TX–phospholipid mixed micelles were separated by centrifugation at 40 000 rpm for 24 h at 4 °C through a continuous 10–30% (w/v) glycerol gradient in 1% TX by using a SW-41 rotor. Twelve-milliliter gradients were poured at 4 °C, and 0.35 mL of a cytochrome *c* oxidase solution (5 mg of protein per mL, 5% TX) was layered on top of the gradient. Separation of the enzyme and mixed micelles by using discontinuous glycerol gradients was accomplished by centrifugation at 35 000 rpm at 4 °C for 14–16 h using a 50.2 Ti rotor. The discontinuous gradients were poured by successively adding 3.5 mL of 50% glycerol and 6.5 mL of 30, 20, and 10%

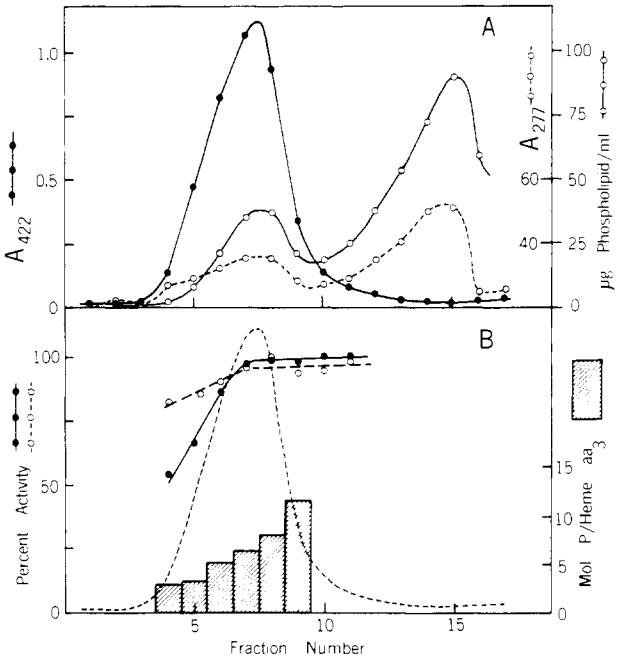

FIGURE 1: Sedimentation of cytochrome *c* oxidase through the first 10–30% glycerol gradient. The sample (0.3 mL) contained 3 mg of cytochrome *c* oxidase and 15 mg of TX. Fractions of 0.73 mL were collected from the bottom of the gradient. Panel A indicates the positions and separation of the cytochrome *c* oxidase–TX–PL complex (●) from the excess TX (O--O) and phospholipid (O—O). Panel B shows the bound phospholipid (bar graph) and the activity of the complex in 0.5% Tween 80 without added phospholipid (●) and with added DPG (O) relative to the position of the cytochrome *c* oxidase complex (---). (See Methods for details.)

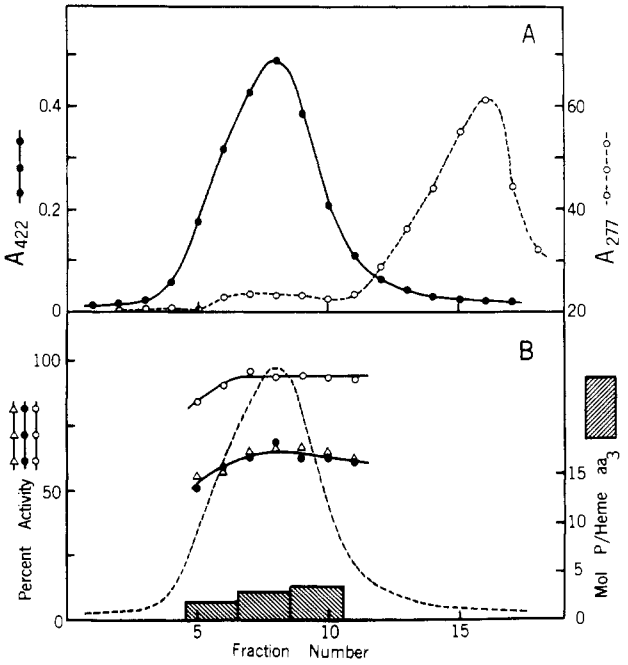

FIGURE 2: Sedimentation of cytochrome *c* oxidase through the second 10–30% glycerol gradient. The sample (0.5 mL) containing 2 mg of cytochrome *c* oxidase and 35 mg TX was obtained by pooling and concentrating fractions 5–7 from the first glycerol gradient (Figure 1). Enzyme pooled from three gradients was loaded on two of the second gradients. Panel A illustrates the separation obtained for the cytochrome *c* oxidase complex (●) and the excess TX (O). Panel B shows the bound phospholipid (bar graph) and the activity of the complex in 0.5% Tween 80 without added phospholipid (●), with added DPG (O), and with added PC (Δ) relative to the position of the cytochrome oxidase complex (---).

glycerol (w/v), each containing 1% TX. All of the gradients contained 0.02 M Tris buffer, 0.09 M NaCl, and 0.1 mM EDTA at pH 8.1. After centrifugation, the gradients were collected by using a peristaltic pump set at 1–2 mL/min after a long needle was carefully lowered to the bottom of the tube.

Results

Removal of Tightly Bound Phospholipids by Triton X-100. Delipidation of cytochrome *c* oxidase by Triton X-100 (TX) was accomplished by incubation of the protein–lipid complex at 5 mg/mL with a large excess of TX (5%) at 5 °C for 30 min. The resulting protein–detergent complex was separated from the PL–TX mixed micelles by sedimentation through a glycerol gradient in 1% TX (Figure 1A). The excess TX, which has a large absorbance at 277 nm, and the extracted phospholipids remained near the top of the gradient and were completely separated from the more dense cytochrome *c* oxidase–TX–PL complex which absorbs at both 422 and 277 nm. The extraction of phospholipids from the complex was not quantitative since 3–12 mol of P remained bound to each heme aa_3 complex. Apparently some of the lipids were extracted from the complex as it moved through the 1% TX in the gradient since the leading edge of the protein peak containing 3–5 mol of P per heme aa_3 was depleted of lipid to a greater extent than the trailing edge of the peak containing 8–12 mol of P per heme aa_3. The importance of the 1% TX in the gradient was further indicated by the fact that sedimentation of a similarly prepared complex through a gradient containing only 1 mM TX (0.064%) did not remove the tightly bound phospholipid since the complex contained 13–16 mol of P per heme aa_3. Further delipidation of the complex was accomplished by pooling the front half of the protein peak from the first gradient (fractions 5–7), concentrating the complex by ultrafiltration, and sedimenting the protein–TX mixture, after adding 6% TX, through a second glycerol gradient in 1% TX (Figure 2A). After this second glycerol gradient, the cytochrome *c* oxidase contained only 2–3.5 mol of P per heme aa_3. Additional cycling of the protein through more TX treatments and glycerol gradients in 1% TX did not result in further lipid depletion of the complex.

Effect of Delipidation upon Electron Transport Activity. Upon removal of all but 2–3 mol of P per heme aa_3, the molecular activity of cytochrome *c* oxidase decreased to 60% of its original level (Figure 2B). The original electron transport activity, measured in 0.5% Tween 80, could be restored by incubation of the complex with DPG in 1% TX as described under Methods, but a similar stimulation of activity with PC was not observed (Figure 2B). The stimulation of activity with DPG was not a general lipid stimulation of cytochrome *c* oxidase activity since cytochrome *c* oxidase containing more than 6 mol of P per heme aa_3 was not stimulated by DPG (Figure 1B). It should be noted that the lipid-depleted enzyme, near the leading edge of the first glycerol gradient, also had low activity that was returned to the original level by DPG (Figure 1B).

Preparative Method for Making Delipidated Cytochrome c Oxidase. A limitation of the method described above was the relatively small amount of delipidated enzyme that could be prepared by using the high-speed swinging bucket rotors (1–2 mg/tube by using a SW-41 rotor). Since large quantities of protein were required for characterization of the small amounts of tightly bound phospholipid, the above procedure was adapted for the use of a large-capacity, high-speed, fixed-angle rotor (50.2 Ti) by using discontinuous glycerol gradients in 1% TX. The results obtained using this preparative method were very similar to the results obtained previously

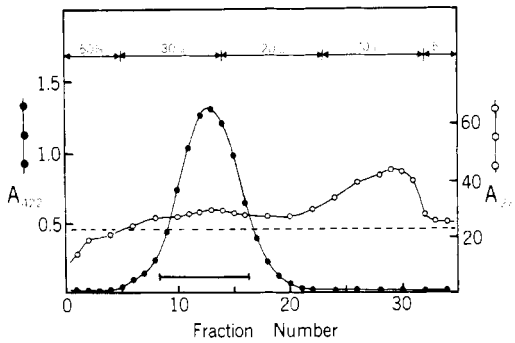

FIGURE 3: Separation of cytochrome *c* oxidase (●) from excess TX (○) by using discontinuous glycerol gradient sedimentation in 1% TX. The sample (1.3 mL) contained 9.1 mg of cytochrome *c* oxidase and 65 mg of TX in 5% glycerol. The positions of the various glycerol layers are shown at the top of the figure above the arrows. The dashed line indicates the A_{277} of 1% TX if it were evenly distributed throughout the gradient. The bar under the protein peak indicates the fractions that were pooled for sedimentation through a second TX–glycerol gradient.

Table II: Activity of Cytochrome *c* Oxidase in Tween 80 during Delipidation by Triton X-100 Using Discontinuous Glycerol Gradients

treatment	mol of P per heme aa_3	act. after addition of different phospholipids[a] none	DPG	PC	PE
none (initial prepn)	28	100[b]	103	95	102
first gradient in 1% TX	5.6	88	109	86	90
second gradient in 1% TX	3.8	63	95	58	68
third gradient in 1 mM TX	3.7	62	94		

[a] Phospholipids were added to cytochrome *c* oxidase in 1% TX, and activities were measured after dilution into 0.5% Tween 80. [b] Activity of the initial preparation was found to be 165 s^{-1} in 0.5% Tween 80 in the absence of added phospholipid. All other activities are expressed relative to this activity.

using the analytical method (compare Figure 3 with Figure 1A). The partially delipidated enzyme was pooled as indicated in Figure 3, concentrated by ultrafiltration, made 5% in TX, and subjected to a second discontinuous glycerol gradient in 1% TX. The results were similar to those shown in Figure 3 except for the lower phospholipid content after the second treatment. The cytochrome *c* oxidase peak was pooled, as it was after the first glycerol gradient (Figure 3), and used for subsequent studies of the delipidated enzyme. One hundred milligrams of delipidated complex having nearly the same low phospholipid content as the material prepared by the continuous glycerol gradient method and stimulation of activity in Tween 80 by DPG but no stimulation of activity in Tween 80 by PC or PE was readily prepared by these two passes through the discontinuous glycerol gradient system (Table II). The delipidated cytochrome *c* oxidase was also assayed in Tween 20 before and after the addition of exogenous phospholipids and compared with the initial activity in Tween 20 in order to assess the possible importance of the detergent used in the activity assays upon the observed phospholipid specificity. The relative activities in Tween 20 were very similar to the relative activities in Tween 80 described above. The initial preparation (28 mol of P per heme aa_3) showed little or no stimulation of activity after incubation with DPG, PC, or PE while the delipidated enzyme (3.8 mol of P per heme aa_3) had decreased activity prior to its incubation with phospholipids (54% of the control), little stimulation of activity by PC or PE, and nearly full regeneration of activity after incubation with DPG (Table III). The 1% TX remaining with the cytochrome *c* oxidase after the second gradient interfered with the analysis of

Table III: Activity of Cytochrome *c* Oxidase in Tween 20 before and after Delipidation by Triton X-100 Using Discontinuous Glycerol Gradients

treatment	mol of P per heme aa_3	act. after addition of different phospholipids[a] none	DPG	PC	PE
none (initial prepn)	28	100[b]	114	101	101
second gradient in 1% TX	3.8	54	90	61	57

[a] Phospholipids were added to cytochrome *c* oxidase in 1% TX, and activities were measured after dilution into 0.5% Tween 20. [b] Activity of the initial preparation was found to be 136 s^{-1} in 0.5% Tween 20 in the absence of added phospholipid. All other activities are expressed relative to this activity.

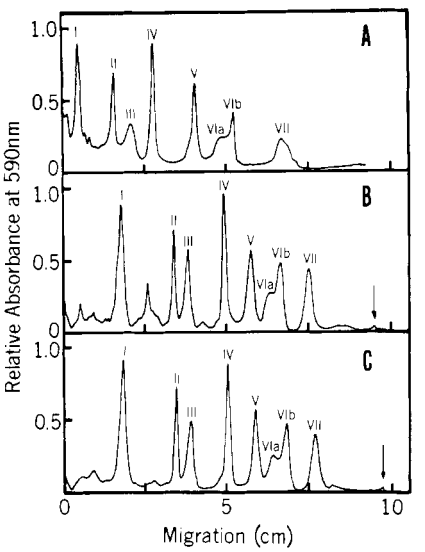

FIGURE 4: Densiometric tracings of sodium dodecyl sulfate–polyacrylamide slab gels after electrophoresis of cytochrome *c* oxidase samples. The observed bands are numbered with Roman numerals according to Downer et al. (1976). (A) Nondelipidated cytochrome *c* oxidase (28 mol of P per heme aa_3) analyzed on a 12.5% polyacrylamide gel in 8 M urea according to Swank & Munkres (1971). The time of electrophoresis was approximately twice the time necessary for the dye to reach the bottom of the gel. (B) Nondelipidated cytochrome *c* oxidase (28 mol of P per heme aa_3) analyzed on a 15% polyacrylamide gel in 2 M urea by using a stacking gel system (see Methods for experimental details). (C) Delipidated cytochrome *c* oxidase (3.8 mol of P per heme aa_3) analyzed by using the same electrophoresis system described above for panel B. The arrows in panels B and C indicate the position of the tracking dye.

phospholipid described below; therefore, the excess TX was generally removed by a third discontinuous glycerol gradient in 0.06% TX, a procedure that effectively removed most of the excess TX, kept the complex in solution, and did not further affect the phospholipid content or activity before or after incubation with phospholipids (see Table II).

Subunit Composition of Delipidated Cytochrome c Oxidase. The subunit composition of the delipidated complex was determined by sodium dodecyl sulfate gel electrophoresis using a stacking system in which 2 M urea had been added to the running gel. This system was found to give better resolution, sharper protein bands, and a flatter base line than the system of Swank & Munkres (1971) and that Downer et al. (1975) used to separate the seven Coomassie blue staining bands of cytochrome *c* oxidase. A comparison of the results obtained for nondelipidated cytochrome *c* oxidase using each method is shown in Figure 4, panels A and B. The stacking gel system clearly separated subunits II and III, proteins that have previously been separated only by the Swank & Munkres procedure, and allowed assessment of the small amounts of con-

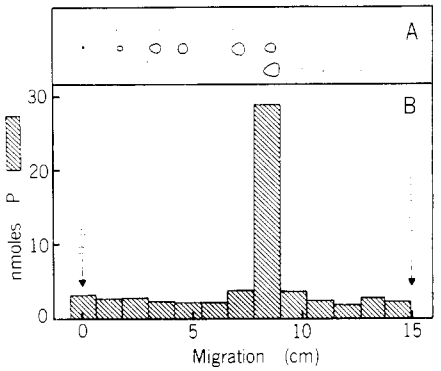

FIGURE 5: Analysis of the phospholipid tightly bound to cytochrome *c* oxidase by thin-layer chromatography as described under Methods. Panel A illustrates the positions of the phospholipid standards as visualized by H_2SO_4 charring and the cytochrome *c* oxidase–phospholipids before being scraped and analyzed for P content. The dotted spots were barely visible after charring. Panel B indicates the P content of fractions that were scraped off of the silica gel plate after chromatography of the phospholipids extracted from 3.2 mg of delipidated cytochrome *c* oxidase (see Methods for details).

taminating protein in the high molecular weight range, i.e., the additional bands between subunits I and II, between subunits III and IV, and above band I that were difficult to observe with the Swank & Munkres system.

Analysis of the delipidated complex by the stacking gel system (Figure 4C) indicated that the TX-glycerol treatment had not removed any of the seven major bands present in the initial preparation since the relative intensities of these bands were unchanged by delipidation (compare panels B and C of Figure 4). In addition, most of the minor contaminants that were present in the initial preparation were removed by the TX procedure. Band VIa, which previously had not been included as one of the cytochrome *c* oxidase subunits due to its lesser staining intensity (Downer et al., 1976), was still present after delipidation. Band VIa, therefore, must be tightly associated with cytochrome *c* oxidase and may be an additional subunit that stains poorly with Coomassie blue.

Phospholipid Composition of Delipidated Cytochrome c Oxidase. The phospholipid composition of the delipidated cytochrome *c* oxidase was determined by thin-layer chromatography on silica gel after extraction of the lipids from the complex as described under Methods. The thin-layer plate was charred with H_2SO_4 to visualize the lipids, a procedure that did not affect the yield of phosphorus. The silica gel scraped from the plates was analyzed for phosphorus following digestion with perchloric acid. DPG accounted for 90–95% of the phospholipid that remained tightly associated with the delipidated enzyme, representing 1.9–2.0 mol of DPG per heme aa_3 (Figure 5). The barely visible spot migrating just before DPG is a small amount of PE, but it represents less than 5% of the total P. The other small spot that migrated ~2.5 cm from the origin (Figure 5A) did not contain P, was colored, and is probably due to heme *a* that was extracted by the chloroform–methanol.

Fatty Acid Composition of the Tightly Bound DPG. The fatty acid composition of the DPG that could not be extracted by TX was determined by gas–liquid chromatography after isolation of the DPG by thin-layer chromatography and preparation of the fatty acid methyl esters as described under Methods. Linoleic acid (18:2) was the predominant fatty acid of the tightly bound DPG. The fatty acid composition was identical, within experimental error, with the composition determined for DPG extracted from whole mitochondrial membranes and purified by thin-layer chromatography (Table IV).

Table IV: Comparison of the Fatty Acid Composition of Phospholipid-Depleted Cytochrome *c* Oxidase with Mitochondrial DPG

	% fatty acid				
sample	16:0	18:0	18:1	18:2	others
DPG bound to cytochrome *c* oxidase[a]	15	4	17	63	
mitochondrial DPG	18	5	13	64	1

[a] Sample contained 3.7 mol of P per mol of heme aa_3.

Discussion

Several conclusions can be drawn from the data presented in this study concerning the functional requirements for phospholipids at the boundary layer of cytochrome *c* oxidase. First, of the ~50 molecules of phospholipid that have been shown to be at the boundary layer of the enzyme using spin-label techniques (Jost et al., 1973, 1977), 90% can be replaced with detergents containing long unsaturated fatty acids, i.e., Tween 80, without affecting the electron transport activity of the complex. This is evident from the data presented in Figure 1, where cytochrome *c* oxidase, containing as little as 5–6 mol of P per heme aa_3 complex, retains full electron transport activity after the bound TX is replaced with Tween 80. Because the tightly bound phospholipid is at least 90% DPG, the fully active enzyme contains a maximum of 2–3 DPG molecules/heme aa_3 complex. Previously, by use of TX exchange and gel filtration methods, ~10 molecules of phospholipid remained bound to the enzyme, 4 or 5 of which were DPG and the remainder of which were approximately equal molar amounts of PE, PC, and lysophospholipids (Robinson & Capaldi, 1977). Apparently, two of these DPG molecules as well as the PE, PC, and lysophospholipids are not required for full activity.

Second, the two or three molecules of DPG that are necessary for maximum activity bind more tightly to cytochrome *c* oxidase than either TX or PC and therefore occupy high-affinity sites on the enzyme. This is evident since very high concentrations of TX are required to remove the bound DPG and even 5% TX treatment leaves at least one molecule of DPG bound to the complex (refer to Figure 2). In addition, the activity of cytochrome *c* oxidase, partially depleted of DPG at these high-affinity sites, is stimulated by exogenous DPG even in the presence of 1% TX (Figure 2, Table II, and Table III). Presumably, even though the cytochrome *c* oxidase is saturated with TX and the exogenous DPG is dissolved in TX micelles, the enzyme selectively extracts the DPG from the TX micelles and regenerates full activity. It is not possible at present to measure whether TX is bound at the high-affinity sites when the DPG is removed, but clearly the binding constant for DPG must be much greater than that for TX in order for the complex to reassociate with DPG in the presence of excess detergent. PC must also bind much less tightly to these sites than DPG since Vik & Capaldi (1977) have shown that excess PC is unable to displace the bound DPG.

Third, the high-affinity phospholipid sites must be occupied by DPG to achieve full activity. Enzyme containing as little as 1 DPG molecule/heme aa_3 complex can be prepared as shown in Figure 2, but all preparations containing less than 5–6 mol of P per complex have diminished activity in Tween 80 or Tween 20 (refer to Figure 2, Table II, and Table III). Regeneration of full activity is possible in either Tween 80 or Tween 20 by the addition of DPG in 1% TX, but no change in activity is observed with PC or PE. Whether PC, PE, Tween

80, or Tween 20 can bind to the high-affinity sites once the DPG is removed by TX is not known, but from the data presented in Figure 2, Table II, and Table III, it is evident that if PC, PE, Tween 80, or Tween 20 does bind, it is unable to regenerate full activity. Evidently, only DPG can meet the functional requirements at these high-affinity sites.

These conclusions on the detergent and phospholipid specificity of the delipidated cytochrome *c* oxidase are quite different than the conclusions of Yu et al. (1975), who studied the cholate-solubilized enzyme. They concluded that Tween 80 and Emasol 1130 (chemically similar to Tween 20) were both able to stimulate the activity of their delipidated enzyme, that a variety of phospholipids including DPG were unable to further stimulate the Tween 80 activity, and that a variety of phospholipids were able to stimulate the Emasol 1130 activity. Our experiments, however, were performed quite differently from theirs since all of our phospholipids were added to the TX-solubilized enzyme while Yu et al. (1975) added their phospholipids to the Tween 80 and Emasol 1130 enzyme. We have found that delipidated cytochrome *c* oxidase loses activity and becomes aggregated when it is incubated with Tween 80 for longer than a few minutes, which may explain the quite different observations of Yu et al. (1975).

Fourth, the high affinity of cytochrome *c* oxidase for DPG is not dependent upon the presence of unusual fatty acids, but rather the enzyme must recognize the unusual structure of this phospholipid or recognize the glycerol head group. Although the bound DPG contains a large percentage of linoleic acid, the average composition of the bound DPG is identical with the composition of DPG purified from whole mitochondrial membranes, suggesting that the DPG occupying the high-affinity sites of cytochrome *c* oxidase is randomly selected from the mitochondrial membrane DPG pool. This result is similar to the conclusions reached by Awasthi et al. (1971) when they analyzed the fatty acid composition of the DPG associated with cytochrome *c* oxidase containing 10 molecules of lipid per complex.

Finally, the tightly bound DPG probably occupies high-affinity binding sites on the surface of cytochrome *c* oxidase rather than sites buried within the complex since one-half to two-thirds of the functionally important DPG can be extracted with 5% TX. It is possible that all of the essential DPG molecules are not equivalent, with one DPG being buried within the complex and inaccessible to TX micelles (this DPG would be the one still associated with the complex in our most delipidated samples) while the other one or two essential DPG molecules are tightly bound to the surface of the complex (these DPG molecules would be the ones that can be extracted by 5% TX). However, due to the distribution of phospholipid remaining after the second gradient, 1 mol of DPG per heme aa_3 at the leading edge of the protein peak and 2 mol of DPG per heme aa_3 at the trailing edge (refer to Figure 2B), it appears more likely that the TX extraction of DPG is incomplete and that all of the essential DPG molecules are equivalent and bound at the surface of the complex.

Although the present procedure was developed as a method of mild delipidation, an interesting consequence of these experiments was the highly purified cytochrome *c* oxidase that was obtained. Most of the minor contaminants present in the original preparation were removed by the TX–glycerol treatment. In addition, the amount of band VIa, a protein often found in cytochrome *c* oxidase preparations (Downer et al., 1976), was not altered by the TX procedure. This raises the possibility that band VIa is not a contaminating protein but rather an additional cytochrome *c* oxidase subunit that stains poorly with Coomassie blue.

In summary, the 50–55 phospholipid molecules comprising the boundary layer of the cytochrome *c* oxidase complex can be divided into at least three classes: (1) phospholipids that easily exchange with nondenaturing detergents and are not essential for activity (~40 mol of P per heme aa_3); (2) tightly bound phospholipids that easily exchange with detergents and are not essential for activity; this group contains 10 mol of P per heme aa_3 and contains ~2 mol each of DPG, PE, PC, and lysophospholipids; (3) tightly bound phospholipids that are essential for electron transport activity but that can be partially extracted by high concentrations of TX; this class of lipids contains 2–3 mol of DPG per heme aa_3.

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