One to two molecules of tightly bound cardiolipin are associated with resolved fractions of cytochrome oxidase containing subunits I to III or I to IV. Large scale isolation of subunits I to IV indicates the presence of approximately 0.5 molecule of cardiolipin per molecule of subunit I. Lipoprotein staining of sodium dodecyl sulfate/urea/acrylamide gels of cytochrome oxidase support the findings that subunit I is a lipoprotein. The resistance of this tightly bound cardiolipin to organic solvent extraction suggests a specific association of some tenacity with the protein.

In contrast to the more general requirement of phospholipid and detergents for cytochrome oxidase (EC 1.9.3.1) activity (1), this enzyme has been shown to have an absolute catalytic requirement for cardiolipin (2, 3). Past studies have indicated the relatively high proportion of cardiolipin in the isolated beef heart cytochrome oxidase (4), the presence of residual cardiolipin following lipid depletion of the enzyme (5, 6), and the resistance of this tightly bound cardiolipin to extraction with chloroform/methanol (2:1, v/v) (7) or cleavage by phospholipase A (8). Tightly bound cardiolipin in cytochrome oxidase is not removed by many solvents that extract loosely bound lipids but can be extracted with alkaline chloroform/methanol (8) or acetone/ethanol mixtures (9), as well as by exchange with the detergent Triton X-100 (2, 3). The conclusion to be drawn from such studies is that cardiolipin plays an essential functional and possibly structural role in the cytochrome oxidase complex. The difficulty of extraction would suggest that the 1 to 2 molecules of tightly bound cardiolipin are either covalently linked to the enzyme or deeply buried among the polypeptides of the enzyme complex. The studies reported in this communication represent an attempt to identify the location of cardiolipin in the cytochrome oxidase complex. Essentially two approaches to this problem have been taken: isolation of purified cytochrome oxidase subunits and characterization of any associated phospholipid, and the partial resolution of the enzyme complex with identification of that fraction containing tightly bound cardiolipin that is resistant to solvent extraction.

METHODS

Preparation of cytochrome oxidase and sodium dodecyl sulfate/urea/gel electrophoresis were as previously described (10). The biuret method (11) was used to determine protein with bovine serum albumin as the standard. Protein samples were solubilized with deoxycholate, and where necessary, sonication or addition of alkali (KOH) was used to compel solubilization. Phospholipid content was calculated from an analysis of total phosphorus (12) and by assuming 4% phosphorus content in phospholipids. Identification of extracted phospholipids was made by bidimensional thin layer chromatography on 0.25-mm-thick Silica Gel 50 on glass plates according to Awasthi et al. (7). Chromatographed extracts were visualized by spraying with phosphorous-specific spray "Phospray" (Supelco, Inc.).

The following procedures were used for the staining of sodium dodecyl sulfate/urea gels with lipoprotein stains, adapted from histochemical methods (13). Approximately 200 µg of cytochrome oxidase protein was electrophoresed per gel. Staining for protein was made with 0.2% Coomassie blue R-250 as previously described (10). For lipoprotein staining, gels were first fixed in a solution of 10% acetic acid containing 1% trichloroacetic acid for 4 h at room temperature, immediately following electrophoresis. Nile blue A (Fisher Scientific Co.) was extracted in methanol (80:20, v/v); the precipitated gels were washed in this solution for 30 min at 70°C. Staining for lipoprotein by the acid hematein technique (13) was performed as follows. Following electrophoresis, gels were rinsed in distilled water before lyophilization of protein samples. The protein precipitate was washed twice with acetone/methanol (28:1), resuspended in 100 µl of distilled water, and evaporated under vacuum to remove traces of methanol and chloroform before lyophilization of protein samples.

Subunits I, II, III, and IV of cytochrome oxidase were purified after dissociation and elution in 2% sodium dodecyl sulfate on a Bio-Gel P-60 column (5 x 180 cm), essentially according to Steffens and Buse (14). Approximately 300 µg of cytochrome oxidase protein was loaded onto the column. To 0.8 volume of the collected aqueous protein fractions in sodium dodecyl sulfate was added 2.0 volumes of methanol and 1.0 volume of chloroform. The resulting homogeneous solutions were stirred at room temperature for 1 h and centrifuged to remove the precipitated subunit fractions. The protein precipitate was washed once by suspension in and centrifugation from 100 µl of methanol/chloroform/water (2:1:0.8), resuspended in 100 µl of distilled water, and evaporated under vacuum to remove traces of methanol and chloroform before lyophilization of protein samples.

An acid-insoluble fraction of cytochrome oxidase (subunits I to III) and acid-soluble fraction (subunits IV to VII) were obtained by resolution in an acidic butanol/methanol mixture (10). An insoluble fraction of cytochrome oxidase (subunits I to IV) and soluble fraction (subunits V to VII) were obtained by resolution in 1% acetic acid. This method of resolution has been fully described elsewhere (10) and involves extraction of the washed and dialyzed particulate cytochrome oxidase in 1% acetic acid, the smaller subunits of the enzyme (V to VII) being selectively solubilized in this medium.Resolved fractions of cytochrome oxidase were thoroughly washed by homogenization in distilled water before lyophilization.

Purified protein subunits or resolved fractions of cytochrome oxidase were subjected to a series of organic solvent extractions. Firstly, approximately 10 mg of lyophilized protein was added to stopped glass tubes and vortexed continually for 1 h at room temperature with 1 ml of acetone. After removal of the acetone, protein was similarly extracted in 1 ml of chloroform/methanol (2:1) and then again in 1 ml of chloroform/methanol/NH₄OH 20% (100:50:1.5), the phosphorus
content of the protein residue being measured at each stage of the extractions. After the final extraction in alkaline chloroform/methanol, 1 ml of ethanol was added to this organic mixture to precipitate the small amount of protein that was invariably solubilized in this alkaline mixture.

RESULTS AND DISCUSSION

When gels that had been heavily loaded with cytochrome oxidase (200 µg of protein/gel) were stained for lipoprotein, all three of the lipoprotein stains used stained that band corresponding in position to subunit I (Fig. 1). In addition to staining for subunit I, Nile blue and Sudan black dyes stained for subunit IV, while acid hematein stained for subunits III and IV. No lipoprotein stain was found at the gel origin or toward the lower end of the gel where protein-free lipid might be expected to migrate (16). If the dyes used in these studies were specifically staining for lipoprotein, then the association of protein and lipid must clearly be of some tenacity, considering the conditions of gel electrophoresis and the procedures used for fixing, staining, and destaining of the gels. However, these stains are not considered to be of absolute specificity for lipoprotein (13) and it was possible that staining could have resulted from other causes, for example, the high hydrophobicity of the larger subunits of cytochrome oxidase (17).

In an attempt to identify the localization of tightly bound cardiolipin in cytochrome oxidase, resolved fractions of this enzyme were subjected to organic solvent extraction. Densitometric gel profiles of the resolved fractions used in these studies are shown in Fig. 2. The results of organic solvent extraction on these fractions are tabulated in Table I. The phosphorus content of those fractions containing the smaller subunits of cytochrome oxidase (either IV to VII or V to VII) was readily extracted in acetone, and, following extraction in chloroform/methanol (2:1), the phosphorus content of such fractions was undetectable. In contrast, following extraction with acetone and chloroform/methanol (2:1), a small but significant phosphorus content remained associated with those fractions containing the larger subunits of cytochrome oxidase (I to III or I to IV). Only after extraction with alkaline chloroform/methanol was this residual phosphorus content completely removed (Table I). Thin layer chromatographic analysis of this alkaline extract confirmed the exclusive presence of cardiolipin (cf. Fig. 4).

A large scale isolation procedure on Bio-Gel P-60 was used to obtain essentially purified subunits I, II, III, and IV of cytochrome oxidase (Fig. 3). Subunit I isolated by this procedure was essentially free of other subunits, while subunit II was about 20% contaminated with subunit I (calculated from their relative gel peak areas). Following removal of sodium
dodecyl sulfate with precipitation of protein from methanol/chloroform/water (2:1:0.8), subunits were lyophilized and then analyzed for phosphorus content (Table II). Subunit I was found to contain approximately 0.9 µg of phosphorus/mg of protein and subunits II and III approximately 0.4 and 0.2 µg of phosphorus/mg of protein, respectively. The phosphorus associated with these subunits was resistant to extraction with acetone or chloroform/methanol but was removed in alkaline chloroform/methanol (Table I). The alkaline extracts from subunits I, II, and III were analyzed by thin layer chromatography that confirmed cardiolipin associated with these subunits (Fig. 4). The cardiolipin extracted from subunit I chromatographed identically to the beef heart cardiolipin standard used, as did that tightly bound cardiolipin extracted under alkaline conditions from resolved fractions of the larger subunits of cytochrome oxidase. Assuming a molecular weight of about 40,000 for subunit I (18), approximately 0.5 mol of cardiolipin/mol of subunit I remain associated following purification of this subunit. This calculation is based on the assumption that the phosphorus content measured in subunit I is wholly accounted for by the cardiolipin content of this subunit. This was confirmed to be the case by chromatographing an alkaline extract of a known amount of subunit I protein and analyzing the phosphorus content of the chromatogram (by scraping off the cardiolipin spot); this amount agreed within 5% with the value for the phosphorus extractable from the same sample weight of lyophilized subunit I.

Studies in our laboratory have confirmed the absolute catalytic requirement for cardiolipin in cytochrome oxidase (2). A minimum of some 2 molecules of cardiolipin per complex of cytochrome oxidase are necessary for the full potential activity of this enzyme; depletion of the cardiolipin content below this level results in a parallel decrease in enzymic activity. The present studies have shown that even after isolation of subunit I from sodium dodecyl sulfate, some 0.5 molecule of this tightly bound cardiolipin can be accounted for in its association with this subunit.

All of the procedures used in these studies in an attempt to

![Fig. 3. Sodium dodecyl sulfate/urea/acrylamide gels of purified cytochrome oxidase subunits. A, subunit I; B, subunit II; C, subunit III; D, subunit IV. Approximately 50 µg of protein were electrophoresed per gel.](image)

![Fig. 4. Bidimensional thin layer chromatography of a mitochondrial phospholipid fraction (A) and phospholipid extract of subunit I (extracted in alkaline chloroform/methanol) (B). Solvent systems were chloroform/methanol/water/28% NH₄OH (130:70:8:0.5) followed by chloroform/methanol/acetone/acidic acid/water (100:20:40:20:10). Spots were visualized by charring with 50% aqueous H₂SO₄, and their phosphorus content was confirmed by spraying with "Phospray."](image)
Localization of Cardiolipin in Cytochrome Oxidase

determine the localization of cardiolipin in cytochrome oxidase might normally be expected to dissociate or extract loosely bound phospholipids. For this reason, we feel it is unlikely that the association of tightly bound cardiolipin with the larger subunits of cytochrome oxidase (and in particular subunit I) is merely a happenstance. There have been numerous reports on the isolation and purification of cytochrome oxidase subunits, primarily from the standpoint of determining amino acid sequences or of identifying bound prosthetic groups (heme and copper) but apparently the lipoprotein nature of some of these subunits had been overlooked. The present studies suggest a major portion of the tightly bound cardiolipin is associated with subunit I, although we cannot rule out the possibility of association with other subunits (most likely subunits II and III); in the unresolved enzyme, cardiolipin might be associated between subunits I and IV.

In our studies on the resolution of cytochrome oxidase (15), we have shown that a subunit I-IV complex, containing all the heme and copper of the original enzyme in a spectrally unaltered state, may represent the limit of resolution of cytochrome oxidase that remains functionally indistinguishable from the isolated soluble holoenzyme. These studies emphasized the importance of subunit IV in retaining the integrity of this complex: removal of subunit IV from the I-IV complex resulted in loss of heme and copper, changes in their spectral characteristics, and loss of enzymic activity. It is significant therefore, that tightly bound catalytic cardiolipin (2) should be found in this fraction (see Table I). The finding of some 0.5 molecule of cardiolipin associated with purified subunit I may mean that a major portion of the 2 molecules of catalytic cardiolipin bound to the enzyme complex are simply lost during the isolation procedure, or that 1 molecule of cardiolipin is more labile in its association with the complex and is more readily extracted upon resolution of the enzyme (e.g. upon resolution of subunit IV from the I-IV complex).

Given the catalytic requirement for cardiolipin in cytochrome oxidase (2), its mode of association with the mitochondrially synthesized subunits of this enzyme (20) and its role therein will clearly be of great importance in finally understanding the mechanism of action of cytochrome oxidase.

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