Subunit Composition of Cytochrome c Oxidase in Mitochondria of Zea mays

Malcolm J. Hawkesford, Andrew D. Liddell, and Christopher J. Leaver

University of Edinburgh, Department of Botany, The Kings Building's, Mayfield Road, Edinburgh, EH9 3JH, Scotland

ABSTRACT

Cytochrome c oxidase has been purified from Zea mays mitochondria by a solubilization with dodecyl maltoside followed by a simple and rapid two step fast protein liquid chromatographic method involving anion exchange on Mono Q and size exclusion chromatography on Superose 12. The preparation obtained was resolved by urea sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a subunit composition comprising polypeptides of apparent molecular masses of 48, 31, and 25 kilodaltons at least one at 16 and 11 kilodaltons and three subunits below 10 kilodaltons. Comparison with a purified yeast cytochrome c oxidase revealed that the four largest subunits showed similar electrophoretic mobilities. Subunits I and II cross-reacted with antibodies raised against the yeast homologous polypeptides. Polypeptides of the plant ubiquinone:cytochrome c reductase complex have also been identified by cross-reaction with antibodies raised against yeast cytochrome b and c1, subunits and by inference from cormigation.

The changing demands for energy and biosynthetic intermediates during plant growth and development are accomodated to a large extent by changes in the number and activity of mitochondria. The elucidation of the mechanisms that control mitochondrial biogenesis and the differentiation of mitochondrial function depends upon a detailed knowledge of the molecular biology and the biochemistry of plant mitochondria (for a thorough review, see ref. 5). This laboratory has sought to clarify details of mitochondrial gene structure and expression and the polypeptide composition of plant mitochondrial respiratory complexes, including the F1, F0 ATPase (8), the ATP/ADP translocator, and more recently the Cyt c oxidase and ubiquinone Cyt c reductase complexes (9). Further interest in plant Cyt c oxidase has arisen from the observation that genome rearrangements in a cytoplasmic male sterile mutant of Sorghum have led to the synthesis of a larger form of the Cyt c oxidase subunit I polypeptide (2). Characterization of the structure and activity of plant Cyt c oxidase during plant development is required to establish the possibility of a causal relationship between this mutation and the cytoplasmic male sterile phenotype.

The Cyt c oxidase complex is the terminal electron acceptor of the mitochondrial inner membrane respiratory chain. The enzyme has been isolated and extensively characterized from a variety of sources (see reviews in refs. 4, 25, 26). In all eukaryotes there are three large subunits, termed I, II, and III, whose genes are located in the mitochondrial genome. These have been isolated and sequenced from yeast, fungal, and mammalian sources (see references in ref. 25) and for plants (7, 10, 11, 19). The polypeptide sequences predicted from the DNA sequences show considerable interspecies homology especially with respect to residues proposed to be involved in prosthetic group binding. The observed variation in Cyt c oxidase between species and preparations is due to the number and composition of nuclear encoded subunits, and it is uncertain whether all reported subunits are essential components of the functional enzyme (14, 24). The functions of these subunits are uncertain but are probably both regulatory and structural and may be required for assembly of the functional complex (6, 12). Isolated enzyme preparations typically consist in total of 2 to 3 subunits in prokaryotes (16, 23), 7–9 subunits in yeast (22), and up to 13 subunits in the mammalian enzyme (13). With the exception of the preliminary work of Asahi et al. (1), who have isolated enzyme preparations from sweet potato and pea, the structure and composition of the plant Cyt c oxidase complex have not been described. Further analysis of the plant enzyme is required and may provide important information applicable to the understanding of the roles of some of the subunits in, for example, the complex mammalian enzyme. The comprehensive analysis of plant Cyt c oxidase and indeed other plant mitochondrial enzymes has greatly hindered by the difficulty of obtaining sufficient quantities of starting material. Typically, only a few hundred milligrams of mitochondrial protein can be easily obtained from a workable amount of plant tissue. A preparation of Cyt c oxidase from sweet potato consisted of five subunits with apparent molecular masses of 39, 33.5, 26, 20, and 5.7 kD (17). The 5.7 kD polypeptide was further resolved to comprise subunits 7.4, 6.8, and 6.2 kD (1, 21), and the 20 kD subunit was resolved into an apparent doublet (1). A five subunit Cyt c oxidase from pea has been purified by the same group (18) and consisted of subunits of molecular mass 39, 33, 28.5, 16.5, and one of 8–6 kD. Immunoprecipitation of Cyt c oxidase from a partially purified pea cytochrome oxidase preparation using an antibody raised against a more highly purified 5 subunit pea Cyt c oxidase preparation precipitated two additional subunits at 13 and 10 kD.

The aim of the work described in this paper was to characterize the Cyt c oxidase subunit composition of maize...
compared to the enzyme from other sources. A Cyt c oxidase preparation has been purified from etiolated coleoptiles using a two-step, high resolution chromatographic procedure. Polypeptides that copurify with the maize Cyt c oxidase enzyme activity have been compared directly with a yeast Cyt c oxidase preparation, and the cross-reactivities of a number of antibodies prepared against yeast and sweet potato Cyt c oxidase subunits were assessed. Information on the polypeptide structure of maize Cyt c oxidase will complement our knowledge of the structure and sequence of the corresponding genes which have been isolated, predominantly from maize, in this laboratory and others (7, 10, 11, 19). The availability of both gene and antibody probes for the components of Cyt c oxidase will allow a detailed analysis of the regulation of expression of this important enzyme during plant development.

**MATERIALS AND METHODS**

**Mitochondrial Membrane Preparation and Solubilization**

Mitochondria from 4 d old, dark-grown, etiolated coleoptiles of maize (*Zea mays* L.) were isolated as described previously, and purified on sucrose gradients (15). Labeling of translation products with 35S methionine in the isolated mitochondria was performed as previously described (15). Mitochondria were stored as a pellet at −80°C. All subsequent steps were performed as rapidly as possible, preferably within one working day.

Membranes were prepared by osmotic shock of the thawed mitochondria (typically 100 mg) in 20 volumes of 20 mM Mops/NaOH (pH 7.2) buffer containing 1 mM EDTA and 2 mM PMSF. Additionally, the suspension was sonicated for 50 s at full power in 10 s bursts with a MSE Soniprep with a standard probe. Unbroken mitochondria and larger fragments were sedimented at 10,000g for 10 min and discarded. The membranes were collected from the supernatant by centrifugation at 410,000g, for 10 min using a Beckman TL-100 ultracentrifuge. This pellet was resuspended in 20 mM Mops/NaOH (pH 7.2) containing 80 mM dodecyl maltoside (Boehringer Mannheim) and 2 mM PMSF at 10 mg protein mL−1 and stirred on ice for 60 min. Insoluble material was sedimented at 410,000g, for 15 min. The resulting brown supernatant was diluted fivefold with 20 mM Mops/NaOH (pH 7.2) prior to chromatography.

**Anion Exchange Chromatography**

The dodecyl maltoside solubilized fraction (10–20 mg protein) was applied to a Mono Q column (Pharmacia FPLC HR 5/5) equilibrated with 20 mM Mops/NaOH (pH 7.2) containing 5 mM dodecyl maltoside at a flow rate of 1 mL min−1. After the column was washed with a further 5 mL of the buffer, the bulk of the protein was eluted from the column with 20 mL buffer containing 0.2 M NaCl and discarded. The Cyt c oxidase fraction was eluted with a 20 mL gradient of 0.2 to 0.55 M NaCl. Fractions containing the peak of Cyt c oxidase activity (oxidation of Cyt c) were pooled and concentrated 10- to 20-fold in an Aminicon stirred ultrafiltration cell with a 100 kD cutoff membrane at a pressure of 0.15 MPa prior to further fractionation.

**Size Exclusion Chromatography**

Aliquots (0.2 mL) of the Mono Q eluate enriched in Cyt c oxidase were applied to a Superose 12 (Pharmacia FPLC HR30/10) size exclusion column equilibrated with 20 mM Mops/NaOH (pH 7.2) containing 100 mM NaCl and 5 mM dodecyl maltoside at a flow rate of 0.5 mL min−1. Fractions were collected and analyzed by electrophoresis on urea-SDS polyacrylamide gels.

**Cyt c Oxidase Assay**

Column fractions were assayed for Cyt c oxidase activity spectrophotometrically. Aliquots of each fraction were added to 20 mM Mes/NaOH (pH 5.9) containing 1% (w/v) Tween 80 and 10 μM (ascorbate) reduced Cyt c (horse heart, Sigma type III) and the oxidation of the Cyt c was followed at 550 nm. Activity was calculated from the initial rate of decrease in absorbance using an extinction coefficient for Cyt c of 18.7 mm−1 cm−1.

**Spectra**

Spectra were recorded on a Varian DMS 90 dual beam spectrophotometer at a scan speed of 50 nm min−1 at room temperature.

**Urea-SDS-PAGE**

The polypeptide composition of column fractions were analyzed by urea-SDS-PAGE (13). Aliquots of column fractions were denatured in 2% (w/v) SDS in 120 mM Tris/HCl (pH 6.8) at 37°C for 30 min and then electrophoresed in a 0.8 mm thick, 16% (w/v) acrylamide/0.04% (w/v) NN'-methylene bisacrylamide separating gel containing 3.6 M urea (BDH Aristar) and 0.375 M Tris/HCl (pH 8.8) and 13% (w/v) glycerol overlaid and copolymerized with a 3% (w/v) acrylamide/0.04% (w/v) NN'-methylene bisacrylamide stacking gel containing 60 mM Tris/HCl (pH 6.8). Standard proteins used were bovine serum albumin (66 kD), catalase (57.5 kD), aldolase (40 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD), myoglobin (17 kD), and Cyt c (12 kD). After electrophoresis, gels were silver stained for protein or electroblotted onto nitrocellulose.

**Silver Staining of Gels**

A procedure modified from Merrill et al. (20) was used. Gels were fixed for 40 min in a solution containing 50% (v/v) methanol, 12% (w/v) trichloroacetic acid, and 2% (w/v) cupric chloride. The gel was then washed in 10% ethanol/5% acetic acid for 20 min prior to a 5 min oxidation step in 0.01% (w/v) potassium permanganate. This was followed by three 10 min washes in 10% (v/v) ethanol/5% (v/v) acetic acid, 10% (v/v) ethanol, and finally distilled water. The gel was then incubated with 0.2% (w/v) silver nitrate for 20 min, followed by brief rinses in distilled water and 10% (w/v) potassium carbonate. The color was developed with 0.02% (v/v) formaldehyde in 2% (w/v) sodium carbonate and stopped with a 10% (v/v) ethanol/5% (v/v) acetic acid wash.
Immunoblotting

Proteins were transferred electrophoretically from 0.8 mm thick polyacrylamide gels onto 0.4 μm nitrocellulose membranes (Schleicher and Schull) in a buffer containing 25 mM Tris/192 mM glycine (pH 8.2), 20% methanol, and 0.1% SDS for 2 h at 0.5 mA. Nitrocellulose filters were then washed in 4 mM KH₂PO₄, 16 mM Na₂HPO₄, 115 mM NaCl, and 0.05% Tween 20 (PBS-Tween buffer) for 2 h with several changes of buffer (3). The filters were then incubated overnight at room temperature in a small volume (typically 20 mL for a 12 × 17 cm filter) of the above buffer containing an appropriate dilution of antisera (100- to 1000-fold) and 0.1% sodium azide, washed in five changes of buffer (10 min washes) to remove excess antisera, and then incubated with 125I-labeled protein A (about 0.1 MBq/20 mL) for 1 h. The excess protein A was washed off with several changes of the PBS-Tween buffer, and the filter was air-dried and autoradiographed using preflashed Dupont Cronex x-ray film. All yeast antibodies were the generous gift of G. Schatz and the sweet potato antibody was kindly provided by T. Asahi.

Protein Assays

Protein was estimated using the Pierce Bicinchoninic acid assay reagent with bovine serum albumin as a standard.

RESULTS

Effective solubilization of mitochondrial membranes prepared as described in Materials and Methods was achieved with 80 mM dodecyl maltoside. Approximately 50% of the membrane protein was solubilized under these conditions (Table I). The soluble extract (410,000g supernatant) was then applied to a Mono Q anion exchange column and part of the elution profile is shown in Figure 1. An initial wash of 0.2 M NaCl removed most of the protein (leaving less than 1%), and Cyt c oxidase containing fractions were then eluted with a gradient of 0.2 to 0.55 M NaCl. Two clearly distinguishable peaks of protein were apparent from the absorbance at 280 nm, the second having a complex profile and containing the bulk of the protein (Fig. 1). The eluted fractions were assayed for Cyt c oxidase activity by measuring the rate of oxidation of reduced Cyt c, and a single peak coincident with the beginning of the major protein peak, and corresponding predominantly to fractions 9 to 12 (0.35-0.42 M NaCl), was observed (Fig. 1, histogram). Recovery of Cyt c oxidase activity at this stage was typically 40 to 50% of the initial activity. Cyt c oxidase activity was not apparent in any other fractions eluting from the column, including the 0.2 M NaCl wash fractions (data not shown). The fractions from the peak containing the Cyt c oxidase activity were then analyzed by urea SDS-PAGE using a procedure effective at resolving subunits of Cyt c oxidase (14). The silver-stained gel of representative fractions eluting between 0.2 and 0.55 M NaCl is shown in Figure 2 (track numbers correspond to 11 of the 18 sequential 1 mL fractions collected across the gradient, of which numbers 7-15 are labeled in Fig. 1 above the columns of the histogram). Fractions 9 through 15 represent the major protein peak and from the polypeptide compositions of the individual fractions it was evident that this was a composite peak of two major overlapping groups of polypeptides. The measured Cyt c oxidation activity suggested that it was only the first of these peaks (fractions 9-12) which contained Cyt c oxidase. Fractions which were coincident with the peak of enzyme activity showed a distinct polypeptide composition with stained bands of apparent molecular masses 48, 31, a 28 kD doublet, 25, 16, 15, an 11 kD minor component, and three to four components below 10 kD. The second group of polypeptides (predominantly fractions 12-14), although overlapping with the Cyt c oxidase peak, showed a distinctive profile with additional polypeptides apparent at 57 kD (a doublet), 38 and 12 kD, and probably at least one below 12 kD. The group of small polypeptides (below 12 kD) was difficult to resolve chromatographically. It is probable that the largest (as indicated by respective migrations) and the two smallest were all coeluting with the Cyt c oxidase peak, while the second largest seemed to coelute only with the second group of polypeptides. The largest and smallest molecular mass polypeptides, although coeluting predominantly with the Cyt c oxidase activity, were also present in the second peak (fractions 12-14) to a greater extent than the third largest polypeptide of this group.

The visible absorption spectra of the air oxidized and the dithionite-reduced enzyme are shown in Figure 3. The characteristic α-band of Cyt c oxidase at 600 nm was clearly visible in the reduced spectrum and allowed the calculation of a haem content of 7 nm mol⁻¹ for the enzyme as eluted from the Mono Q column.

The fractions containing the peak of enzyme activity were pooled, concentrated, and then subjected to size exclusion chromatography on a Superose 12 column. The protein elution profile as represented by the absorbance at 280 nm,

**Table I. Purification of Cyt c Oxidase from Zea mays**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity*</th>
<th>Yield</th>
<th>Specific Activity</th>
<th>Purification</th>
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</thead>
<tbody>
<tr>
<td>Mitochondria*</td>
<td>90</td>
<td>62.25</td>
<td>100</td>
<td>0.69</td>
<td>1</td>
</tr>
<tr>
<td>Membrane particles</td>
<td>52</td>
<td>48</td>
<td>77</td>
<td>0.92</td>
<td>1.3</td>
</tr>
<tr>
<td>Dodecyl maltoside</td>
<td>25.2</td>
<td>56.7</td>
<td>91</td>
<td>2.25</td>
<td>3.3</td>
</tr>
<tr>
<td>Mono Q peak</td>
<td>0.86</td>
<td>28.5</td>
<td>46</td>
<td>33.1</td>
<td>48</td>
</tr>
<tr>
<td>Superose 12 peak</td>
<td>0.40</td>
<td>15.9</td>
<td>26</td>
<td>39.7</td>
<td>58</td>
</tr>
</tbody>
</table>

* Activity was determined in an aliquot of each fraction from the initial rate of oxidation of 10 μM reduced Cyt c, and the total activity was derived from the product of this value and the total fraction volume.  
* The mitochondria were prepared from 700 g of etiolated Zea mays coleoptiles.  
* The calculated turnover number was 106 s⁻¹ using a molecular mass for the enzyme of 160 kD.
Figure 1. Anion exchange chromatography of a maize Cyt c oxidase enriched mitochondrial membrane fraction. A 10 mL fraction containing 20 mg of five times diluted 80 mM dodecyl maltoside solubilized, mitochondrial protein was applied to a Pharmacia FPLC Mono Q column. The protein elution profile (absorbance at 280 nm) and relative Cyt c oxidase activity (measured by the rate of oxidation of reduced Cyt c, see histogram) of 1 mL fractions eluting between 0.2 and 0.55 M NaCl, in 20 mM Mops/NaOH (pH 7.2) containing 5 mM dodecyl maltoside is shown. Fractions 7–15 are numbered.

together with the positions at which standard proteins elute (in the absence of any detergent, marked A-E), is shown in Figure 4. A peak was apparent coincident with the excluded volume, and a major broad peak corresponding to a molecular mass of around 215 kDa, together with a shoulder at 66 kDa, represented the majority of the protein. Fractions were collected and analyzed by urea SDS-PAGE and those corresponding to the major protein peak, fractions 1 to 7 as indicated in Figure 4, are shown in Figure 5. The excluded peak was a composite of all polypeptides and was probably aggregated material (data not shown). The composition of the fractions around 215 kDa were similar to the Cyt c oxidase containing fractions from the Mono Q peak. The 48, 31, 25, 16, and three polypeptides below 10 kDa coeluted as a single group. Cyt c oxidase activity was measured in these fractions, and although there was typically a 50% reduction in the total activity recoverable at this stage, there was an improvement in the specific activity (Table I). The 28 kDa doublet and the faint 11 kDa polypeptides present in the Mono Q Cyt c oxidase preparation were substantially reduced or not detected in the Superose Cyt c oxidase preparation when similar amounts of total protein were analyzed by SDS-PAGE. The later eluting low molecular mass shoulder was enriched in the 28 kDa doublet and the 25 kDa polypeptide. The 25 kDa polypeptide typically eluted from the column as a broad peak beginning in the Cyt c oxidase peak and trailing into the later fractions.

Figure 6 shows a comparison of a purified yeast Cyt c oxidase preparation (lane 1) and the final purified maize Cyt c oxidase as eluted from the Superose 12 column (lane 2). The two Cyt c oxidase preparations show a number of similarities. The four largest polypeptides in the yeast enzyme all have corresponding, similarly migrating polypeptides in the maize preparation. An additional polypeptide around 28 kDa in the maize preparation does not co-purify with the other subunits on the Superose 12 separation (Fig. 5). Two polypeptides at around 10 and 14 kDa in the yeast preparation were not observed in our plant preparations (Figs. 2, 5; and unpublished observations), although there is a faint staining polypeptide at 11 kDa. Three to four small subunits with molecular masses below 10 kDa were consistently found in the maize preparation using this electrophoresis system; however, only one polypeptide in the yeast preparation was apparent.

Cross-reactivity of maize polypeptides to antibodies raised against Cyt c oxidase and ubiquinone: Cyt c reductase subunit polypeptides from other organisms is shown in lanes A to E. A fraction representative of all the polypeptides associated with the complex peak eluting from the Mono Q column, including the Cyt c oxidase polypeptides and the later eluting contaminating polypeptides, was separated by urea-SDS-PAGE. The resulting gel was electroblotted onto nitrocellulose, and the filters were then screened sequentially with a number of cross-reacting heterologous antibodies in order to identify individual polypeptides. Antigen-antibody complexes were visualized with 125I-labeled protein A, and the resulting autoradiograms are shown in lanes A to E. An antibody against yeast Cyt c oxidase subunit I reacted with a polypeptide of apparent molecular mass 48 kDa (lane A) which corresponded with the largest subunit in the purified maize
**Protein coefficient of concentration of enzyme as fraction.**

An aliquot (0.2 mL) of the Cyt c oxidase-enriched fraction from the Mono Q purification was subject to chromatography on a Pharmacia FPLC Superose 12 column and eluted with 20 mM Mops/NaOH (pH 7.2) containing 5 mM dodecyl maltoside and 0.1 M NaCl. Seven fractions (labeled 1-7 and marked by diamonds, •) were collected across the major protein peak for analysis by SDS-PAGE (see Fig. 5). The positions at which standard proteins elute in the absence of detergent are indicated: A, blue dextran (>2 x 106 kDa); B, thyroglobulin (669 kDa); C, ferritin (440 kDa); D, catalase (232 kDa); and E, aldolase (158 kDa).

preparation. This same filter was subsequently probed with an antibody against the 5.7 kDa sweet potato Cyt c oxidase (ref. 17, the Asahi preparation subunit V, lane B). Although this antibody reacted with a component in the region of the smallest polypeptides, it was not possible to distinguish which of the sub-10 kDa polypeptides were cross-reacting. A minor cross-reaction with the 17 kDa subunit also occurred. A replicate filter was labeled first with yeast Cyt c oxidase subunit I antibody and then with an antibody against yeast Cyt b (a subunit of the ubiquinone:Cyt c reductase complex) and this labeled a polypeptide at 37 kDa (lane C). This filter was then labeled with a third antibody raised against yeast Cyt c1 (also a subunit of the ubiquinone:Cyt c reductase complex) which labeled a polypeptide at 32 kDa. The two polypeptides that were detected using these antibodies (37 and 32 kDa) were not present in the final Cyt c oxidase preparation (Figs. 5 and 6, lane 2) but were clearly evident in the latter part of the Cyt c oxidase containing Mono Q peak (Figure 2). The filter in lane E was labeled with Cyt c oxidase subunit I antibody, Cyt b antibody, and yeast Cyt c oxidase subunit II antibody. A polypeptide indicating the presence of Cyt c oxidase subunit II antibody was labeled at 31 kDa, comigrating with the second largest subunit present in the purified enzyme. A number of other antibodies to yeast, bacterial, and mammalian Cyt c oxidase, and ubiquinone:Cyt c reductase complex subunits showed no cross-reactivity to the plant polypeptides (data not shown).

**DISCUSSION**

Several methods for the isolation of Cyt c oxidase from plant material have been evaluated (MJ Hawkesford, unpublished observations); however, the most successful method in
gives polypeptides to determine urea-SDS-PAGE column at one mays Cyt (on Mono Q) proteins by tase II. 11 subunit V Cyt Western strips gel fraction reductase 6. Comparison c 1 HAWKESFORD The separation of other Cyt for the maize oxidase was of the Mono Q separation of the maize oxidase complex (from the Mono Q purification) separated on an identical gel prior to transfer to nitrocellulose membrane. Membrane strips were exposed to cross-reacting antibodies as follows: A to E, Cyt c oxidase subunit I antibody; B, sweet potato Cyt c oxidase subunit V antibody; C to E, Cyt b (from ubiquinone:Cyt c oxidoreduc- tase complex) antibody; D, Cyt c, antibody; and E, Cyt c oxidase subunit II antibody. Identified, cross-reacting subunits are marked and molecular masses of standard proteins are indicated.

our hands was found to be the rapid and simple procedure described.

The separation of solubilized maize mitochondrial mem- brane proteins by high resolution anion exchange chromatography (on Mono Q) has been previously reported (9). Using cross-reacting heterologous antibodies, a number of polypeptide components of respiratory complexes of the inner mem- brane were identified including Cyt c oxidase subunits I and II. The Cyt c oxidase complex eluted from the Mono Q column at a high salt concentration, well resolved from most of the other protein. This separation system has been used as the basis for the purification of maize Cyt c oxidase in order to determine its subunit composition. If sufficient care is taken, a preparation suitable for many purposes is obtained after this step. The further purification on the Superose 12 column removes additional contaminating polypeptides and gives a slight improvement in specific activity. This step has aided in the determination of copurifying subunits of the Zea mays Cyt c oxidase as reported in this paper. Resolution by urea-SDS-PAGE gave a subunit composition comprising polypeptides of apparent molecular masses 48, 31, 25, at least one at 16, 11, and probably three below 10 kD. Table II summarizes the compositions of published plant Cyt c oxidase preparations (including that reported in this paper) together with the apparent molecular masses of the yeast subunits determined in our hands (Fig. 6) and typical published values for the larger bovine Cyt c oxidase subunits (13). The apparent discrepancies, particularly for subunit I, probably reflect the differing PAGE systems used to resolve the subunits. The large discrepancy of the apparent molecular mass of maize subunit I, as compared to that predicted from the gene se- quence, can only be attributed to the anomalous behaviour of this particularly hydrophobic polypeptide during elec- trophoresis. In the absence of urea the discrepancy is even greater (our unpublished data). The 48 and 31 kD polypeptides cross-reacted with antibodies to yeast Cyt c oxidase subunits I and II and may be confidently identified as the homologous plant subunits. The 28 kD doublet observed in the Mono Q preparation was resolved separately on the Superose 12 separation and is, therefore, either a contaminating polypeptide or only a loosely bound subunit. These three polypeptides are almost certainly the three mitochondrially encoded subunits of plant Cyt c oxidase. In contrast, however, on the basis of DCCD binding data, it has been suggested that the sweet potato 20 kD doublet corresponds to subunit III from other sources (1).

Identification of the nuclear encoded components is more difficult. It is likely that one or more components with a molecular mass of approximately 16 kD, and three subunits below 10 kD, are present. The 16 kD subunit exactly comi- grates with the yeast subunit IV (Fig. 6, lanes 1 and 2). Some sequence data will be required to positively establish that these are homologous subunits, although we have not ob- served any antibody cross-reactivity (data not shown). The faint 11 kD polypeptide may be a loosely bound component depleted in this preparation, a proteolysis product or a minor contaminant. Therefore, in our preparation, between one and three subunits occur in the 10 to 20 kD range, which would be consistent with the complexity of the yeast enzyme, but which is in contrast to other reports of plant Cyt c oxidase (1, 17, 18) that failed to identify any polypeptides in this size range. The antibody to plant subunit V used in this study was raised by Maeshima and Asahi (17) against their initial unre- solved 5.7 kD subunit, and although this cross-reacted with our preparation we were unable to resolve which polypeptides were involved in the cross-reactivity. In summary (see Table II), the maize Cyt c oxidase preparation comprises 7 to 9 subunits and is similar, although not identical, in composition to the preparations of Maeshima and Asahi (17). Some variation might be expected due to the vagaries of SDS-PAGE systems or may be attributed to species variation. There is a strong similarity to the yeast preparation and apparently less complexity as compared to reported mammalian enzyme preparations (13).

The cross-reactivity of antibodies raised against yeast Cyt b and c I polypeptides to plant polypeptides eluting from the Mono Q column have enabled the identification of the frac- tion containing the ubiquinone:Cyt c reductase complex. Other co-eluting polypeptides almost certainly represent other subunits of the ubiquinone:Cyt c reductase complex, the 57 kD doublet being the 'core' components, the 38 kD polypeptide, the Cyt b subunit, and an additional probable subunit of 12 kD. Of the four polypeptides observed below 10 kD in the

Figure 6. Comparison of purified Cyt c oxidase from yeast and maize and cross-reactivity of maize polypeptides with antibodies raised against yeast and sweet potato polypeptides. Silver-stained urea-SDS-PAGE of yeast (lane 1, a gift from G Schatz) and maize (lane 2) Cyt c oxidase and autoradiographs (lanes A-E) of immunodecorated Western blots of a partially purified Cyt c oxidase/ubiquinone:Cyt c reductase fraction (from the Mono Q purification) separated on an identical gel prior to transfer to nitrocellulose membrane. Membrane strips were exposed to cross-reacting antibodies as follows: A to E, Cyt c oxidase subunit I antibody; B, sweet potato Cyt c oxidase subunit V antibody; C to E, Cyt b (from ubiquinone:Cyt c oxidoreduc- tase complex) antibody; D, Cyt c, antibody; and E, Cyt c oxidase subunit II antibody. Identified, cross-reacting subunits are marked and molecular masses of standard proteins are indicated.
Mono Q preparation, between one and three may be subunits of the ubiquinone:Cyt c reductase complex. The Cyt c1 subunit was not detected by protein silver staining but was detected immunologically (Fig. 6, lane D). The Rieske iron-sulphur protein and other known components of fungal and mammalian ubiquinone:Cyt c reductase have not been identified.

In conclusion, we have isolated a plant Cyt c oxidase from maize and identified a number of copurifying component polypeptides. Immunological cross-reactivity occurs only between the corresponding subunits I and II of the plant and yeast Cyt c oxidase, although the four largest subunits comigrate on PAGE. Sequence analysis of the genes for the three largest mitochondrially encoded subunits from plants reveals that they share significant homology with the equivalent yeast and mammalian genes. An immediate task must be the determination of the partial N-terminal sequence of the polypeptide we have assigned as subunit III for comparison with the known gene sequence. Additionally, more information, particularly sequence information, is required on the smaller, and presumably nuclear-encoded, polypeptides that have been tentatively identified in this paper as components of plant Cyt c oxidase. Such information will be used in the study of the control and biosynthesis of this essential component of the plant mitochondrial electron transport chain.

LITERATURE CITED


Table II. Summary of Known Subunit Molecular Masses of Plant Cyt c Oxidase Preparations

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sweet Potato</th>
<th>Pea [18]</th>
<th>Zea mays*</th>
<th>Zea mays*&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yeast*</th>
<th>Bovine [13]</th>
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<tbody>
<tr>
<td>II</td>
<td>33.5</td>
<td>33</td>
<td>31</td>
<td>30.8 [7]</td>
<td>30</td>
<td>26</td>
</tr>
<tr>
<td>III</td>
<td>26</td>
<td>28.5</td>
<td>25</td>
<td>29.4 [19]</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>IV</td>
<td>20 (doublet)</td>
<td>16</td>
<td>16 (doublet)</td>
<td>16</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>V–VIII</td>
<td>7.4, 6.8, 6.2</td>
<td>8–6</td>
<td>11 (minor component) and 3–4 polypeptides &lt;10</td>
<td>13, 10 and &lt;10</td>
<td>12 and possibly one</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data in this paper. <sup>b</sup>Predicted from gene sequence. <sup>c</sup>References appear in brackets.

