Structurally Unique Plant Cytochrome *c* Oxidase Isolated from Wheat Germ, a Rich Source of Plant Mitochondrial Enzymes[†,‡]

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ABSTRACT: Purification and characterization of plant cytochrome *c* oxidases have been impeded by the difficulty of obtaining enough plant mitochondria. We have found commercial wheat germ to be a rich and convenient source of mitochondrial membranes containing respiratory chain complexes in ratios and amounts similar to mitochondria prepared from etiolated seedlings. Cytochrome *c* oxidase was purified from these membranes by anion-exchange (MonoQ) fast protein liquid chromatography. The enzyme is highly active (turnover number up to 1000 s^{-1}) and exhibits biphasic cytochrome *c* reaction kinetics similar to those of beef heart oxidase. As with other plant oxidases, the visible spectrum of wheat germ oxidase in the reduced form is blue-shifted compared to other eukaryotic cytochrome oxidases, with peaks at 441 and 602 nm. The electron paramagnetic resonance spectrum of Cu_A of the wheat germ enzyme is very similar to that of the maize and beef heart enzymes, suggesting that the copper environment is not altered. Sodium dodecyl sulfate–polyacrylamide gels show a subunit composition in which subunits I–IV resemble those of the yeast enzyme in size and antigenicity, while three to four smaller peptides are dissimilar to yeast and other eukaryotic oxidases. A difference between the subunit composition of the wheat germ and wheat seedling enzymes suggests the existence of a developmental or tissue-specific form of cytochrome oxidase in plants.

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal electron acceptor in the respiratory chain of the inner mitochondrial membrane. It couples electron transfer from cytochrome *c* to O_2 with the movement of protons across the inner membrane, creating an electrochemical gradient that is utilized to synthesize ATP. The mechanism coupling electron transfer to proton pumping is not well understood; various theories of proton pumping have suggested the involvement of heme *a* (Babcock & Callahan, 1983), Cu_A (Gelles et al., 1986), or the binuclear (heme a_3–Cu_B) center (Wikström, 1989a,b).

All of these metal centers are proposed to be contained in two of the largest subunits of most cytochrome *c* oxidases (Wikström et al., 1985). Mammalian cytochrome oxidase has, however, a total of 13 nonidentical subunits (Kadenbach et al., 1983). In eukaryotes, the three largest are encoded by the mitochondrial DNA, and the remaining are nuclear-encoded (Sebald et al., 1972; Mason & Schatz, 1973; Kolarov et al., 1981). Oxidases such as those from yeast (Power et al., 1984) and plants (Matsuoka et al., 1981; Nakagawa et al., 1987) share similar mitochondrial subunits to the beef heart, but contain dissimilar nuclear subunits. Despite intense study of the yeast and mammalian enzymes, the role of these other subunits is not clear, though tissue-specific (Kadenbach et al., 1986) and developmental (Bisson & Schiavo, 1986) changes in subunit composition suggest a regulatory function.

The plant cytochrome *c* oxidases studied so far, sweet potato (Maeshima & Asahi, 1978; Nakagawa et al., 1987), pea (Matsuoka et al., 1981), and maize (Dutch et al., 1987; Hawkesford et al., 1989), have a subunit composition different from other eukaryotic oxidases, apparently containing only seven or eight peptides. The three mitochondrially encoded subunits of the plant enzyme are similar in sequence and mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] to those of yeast, but the nuclear-encoded subunits are different. The DNA sequences available for the larger subunits indicate that most of the conserved residues are maintained. An important exception is the prediction from the DNA sequence that an arginine replaces the conserved cysteine (228) at the Cu_A site of subunit II in wheat (Bonen et al., 1984), implying altered liganding and functional properties of Cu_A. RNA sequences, however, indicate that editing has restored the cysteine (Covello & Gray, 1989). This has yet to be confirmed by protein sequencing. Plant oxidases also have spectral properties somewhat different from other oxidases: the reduced peaks are shifted from 443 to 441 nm and from 605 to 602 nm, suggesting an altered heme *a* environment (Chance et al., 1968).

The functional significance of the unusual subunit and spectral characteristics of plant cytochrome oxidases has not been established, in part due to the difficulty in obtaining enough plant mitochondria to do thorough enzyme studies, as well as the apparent lability of the oxidase which has been reported to lose much of its activity during purification (Maeshima & Asahi, 1978; Matsuoka et al., 1981; Hawkesford et al., 1989). Etiolated seedlings are frequently used as a starting material to reduce contamination of the mitochondrial fraction with chloroplasts, but this approach is time-consuming and low-yield. The use of wheat germ as an alternative source of plant material provides a substantial advantage in time and yield. Although it has been shown that unimbibed plant embryos do not contain recoverable intact mitochondria and have low amounts of mitochondrial protein

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[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; PVP, poly(vinylpyrrolidone); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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(Sato & Asahi, 1975), we have isolated a membrane fraction from commercial wheat germ that has a protein complement and cytochrome oxidase specific activity similar to etiolated wheat seedling mitochondria. Thus, with this convenient and rich source, we now have the means to purify a highly active cytochrome oxidase suitable for structural and functional analysis.

Experimental Procedures

Materials. Wheat germ was purchased from Star of the West Milling Co. in Frankenmuth, MI, and King Milling Co. in Lowell, MI. Wheat seed (*Triticum aestivus* var. Titan Red) was obtained from Michigan Foundation Seed Co. Maize seed (*Zea mays* variety GL466) was purchased from Great Lakes Hybrids, Inc. Lauryl β-D-maltoside was synthesized according to Rosevear et al. (1980). Cytochrome *c* Sigma type VI was further purified according to Thompson and Ferguson-Miller (1983). Asolectin vesicles were prepared by sonicating 40 mg/mL asolectin (Associated Concentrates, Long Island, NY) for 1 min/mL in 2% cholate and 75 mM K-HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid potassium salt], pH 7.4. Monospecific polyclonal antibodies to yeast cytochrome oxidase subunit IV were the gift of Dr. R. Poyton. Other chemicals were obtained from the sources indicated: BCA protein assay reagent, Pierce Chemical Co.; bovine serum albumin (BSA), Sigma fraction V; ultrapure sucrose, ICN; E-64, Boehringer-Mannheim; acrylamide and *N,N'*-methylenebis(acrylamide), Serva; insoluble poly(vinylpyrrolidone) (PVP), Serva Polyclar AT; and goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (Western blotting grade affinity purified), Bio-Rad Laboratories.

Preparation of Wheat and Maize Seedling Mitochondria. All operations were carried out at 0–4 °C using prechilled detergent-free glassware. Intact wheat mitochondria for use in respiratory control studies were prepared by the method of Day and Hanson (1977) from etiolated seedlings grown in vermiculite for 5 days at 25 °C and 80% relative humidity.

To prepare mitochondrial membranes for cytochrome oxidase isolation, wheat seeds that were soaked in aerated water overnight were spread between two sheets of stiff plastic mesh and suspended over distilled, aerated water. Soaked maize seeds were planted in vermiculite. Seedlings were grown in the dark at 25 °C and 80% relative humidity for 5 days and were harvested by using electric grass shears. The cut seedlings were soaked in cold (4 °C) distilled water for an hour and then drained before grinding 10 s at the highest speed in a commercial Waring blender (Model 34BL22) with 0.4 M mannitol, 50 mM KH_2PO_4, 5 mM EGTA, 0.2% BSA, pH 7.7, and PVP (0.1 g/g seedlings) at approximately 1 mL of buffer/g of seedlings. The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 15 min. The supernatant was recentrifuged at 27000*g* for 20 min to pellet the mitochondria, which were resuspended with a Potter–Elvehjem-type tissue grinder (clearance 0.2 mm) in 0.4 M mannitol, 5 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], and 0.1% BSA, pH 7.2, and centrifuged at 40000*g* for 20 min. The pellet, resuspended in a small volume of 0.35 M mannitol, 5 mM MOPS, 50 mM KCl, and 1 mM EGTA, pH 7.2, was layered onto a 0.6 M sucrose cushion and centrifuged at 40000*g* for 20 min. In this step, the mitochondria are pelleted, while the fluffy yellow contaminants remain in suspension and are easily poured off. The mitochondria were next depleted of cytochrome *c* by resuspending the pellet in a 10-fold volume of distilled water for 1 min (to burst the outer membrane) before adding KCl to a final concentration of 0.15 M. This mixture was centrifuged at 40000*g* for 20 min and the mitochondrial pellet resuspended in the previous mannitol buffer and stored at −80 °C.

Preparation of Wheat Germ Mitochondrial Membranes. Wheat germ (unimbibed) was homogenized for 15 s in a chilled (4 °C or less) buffer (0.4 M sucrose or mannitol, 50 mM KH_2PO_4, 5 mM EGTA, 0.2% BSA, pH 7.0, ±2 mM PMSF, 1 mM EDTA, and 10 μM E-64) using a food processor (Black and Decker Model FP1D) at a ratio of 150 g of wheat germ and 10 g of PVP per liter of buffer. The homogenate was centrifuged at 13000*g* for 15 min, and the supernatant was filtered through 35-μm nylon mesh to remove congealed fat. The filtrate was then centrifuged at 28000*g* for 40 min. The loose pellet was resuspended in buffer (0.4 M sucrose or mannitol, 5 mM TES, 0.1% BSA, pH 7.2 ±2 mM PMSF, and 1 mM EDTA) by hand using a Potter–Elvehjem-type tissue grinder (clearance 0.2 mm) and then centrifuged at 3000*g* for 15 min to remove large debris. The resulting supernatant was filtered through 35-μm nylon mesh to remove congealed fat before centrifuging at 48000*g* for 20 min. The final mitochondrial pellet was resuspended in buffer (0.35 M sucrose or mannitol, 50 mM KCl, 5 mM TES, and 1 mM EGTA, pH 7.2) and stored at −80 °C.

Isolation of Cytochrome Oxidase from Mitochondrial Membranes. Mitochondrial membranes were incubated on ice for 30 min with 2 mg of lauryl maltoside/mg of mitochondrial protein, 10 μg of DNase/mg of protein, 1 mM $MgCl_2$, ±2 mM PMSF, and 10 μM E-64 added to the final suspension buffer. (Proteolytic inhibitors were normally used throughout in the wheat germ but not in the wheat or maize seedling preparations.) The mixture was centrifuged at 40000*g* for 20 min. The supernatant was filtered through a 0.45-μm membrane and loaded onto a Pharmacia MonoQ 10/10 anion-exchange column on the Pharmacia FPLC system at 0.15 M KCl. Typically, 900 mg of starting mitochondrial protein was loaded onto the 10/10 column. A discontinuous gradient (described in the legend of Figure 2) of 0–1.0 M salt in buffer (FPLC buffer: 5% sucrose, 20 mM KH_2PO_4, and 4 mM lauryl maltoside, pH 7.5) was applied to the column at 1 mL/min. The salt concentration was held constant at 0.32 and 0.39 M KCl, just before and just after the oxidase elutes at 0.35 M KCl. This separates the oxidase from both the large preceding peak and the following peak, cytochrome *c* reductase eluting at 0.42 M KCl. The oxidase was concentrated by reloading the enzyme diluted 10-fold onto a MonoQ 5/5 column, washing at 0.3 M KCl to remove contaminants and inactive enzyme, and then eluting it from the inverted column with 0.5 M KCl in FPLC buffer at a very slow flow rate. The enzyme could then be further purified by running concentrated oxidase on a Superose 6 gel filtration column (equilibrated to 0.1 M KCl in FPLC buffer) at a flow rate of 0.2 mL/min.

Cytochrome oxidase was purified from maize mitochondria by a similar procedure except that a continuous (0–1 M KCl in FPLC buffer) gradient was used in the elution, no proteolytic inhibitors were used, and the fractions were sometimes frozen and thawed between purification steps.

Assay Methods. The electron-transfer activity of purified cytochrome oxidase was measured polarographically with a Gilson Model 5\6H oxygraph at 25 °C as described by Ferguson-Miller et al. (1975) except that asolectin vesicles (1.1 mg/mL) and 0.1% lauryl maltoside were added to each assay mixture. Assays for maximal activity were performed in 50 mM KH_2PO_4, pH 6.5, while kinetic assays were performed in 25 mM Tris-cacodylate, pH 7.9. Turnover numbers were calculated as described in Thompson and Ferguson-Miller (1983). Protein concentrations were determined by the bi-

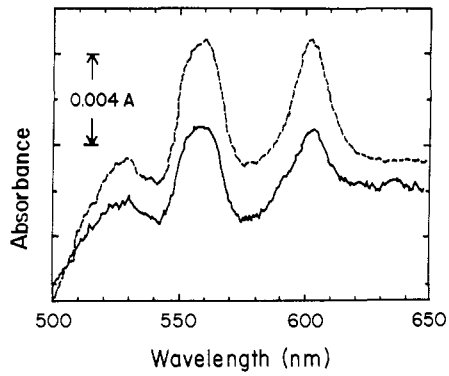

FIGURE 1: Difference spectra (dithionite-reduced minus air-oxidized) of lauryl maltoside solubilized (2 mg/mg of protein) mitochondrial membranes from wheat germ [1.0 mg/mL (---)] and etiolated wheat seedlings [0.32 mg/mL (—)].

cinchoninic acid (BCA) method (Smith et al., 1985).

Spectral Methods. UV and visible spectra were recorded on a Perkin-Elmer Lambda 4B spectrophotometer. The extinction coefficients for absolute spectra of cytochrome aa_3 (dithionite-reduced or air-oxidized) were taken from the spectra published by van Buuren et al. (1972): cytochrome aa_3 (reduced), 605–650 nm = 40 mM^{-1} cm^{-1} and 440–490 nm = 204 mM^{-1} cm^{-1}; cytochrome aa_3 (oxidized), 420–490 nm = 140 mM^{-1} cm^{-1}. EPR spectra were recorded at X-band with a Bruker ER200D with 1-mW microwave power and 10-G modulation amplitude. Samples were kept at 10 K by using an Oxford ESR-9 liquid helium cryostat.

SDS–PAGE of Purified Cytochrome Oxidase. Subunit composition of the oxidase fractions was evaluated by SDS–PAGE (Bio-Rad Protean II, 200 V for 2 h in chilled buffer) using the procedure of Kadenbach et al. (1983) except that 0.04% bromophenol blue was added to the sample buffer. Protein samples were precipitated with 10% trichloroacetic acid (final concentration 4.5%) before resuspension in the sample buffer, and sonicated for 30 min at 15 °C. Protein bands were stained overnight with Coomassie brilliant blue.

Gels used for Western blot analysis were blotted onto nitrocellulose (0.45-μm pore size, Bio-Rad Laboratories) in a buffer containing 12.5 mM Tris, 96 mM glycine, and 20% methanol for 1 h at 100 V. (The filter paper on the cathode side of the gel was soaked in this buffer with 0.5% SDS for 10 min before blotting.) The blots were stained for 5 min with amido black (0.025% in 7.5% acetic acid and 22.5% ethanol) and destained with water. Blots were blocked overnight in a solution of 5% milk powder in 20 mM Tris, 0.5 M NaCl, and 0.05% Tween-20, pH 7.5, and then incubated for 4 h with antibodies to yeast oxidase subunit IV. Goat anti-rabbit IgG alkaline phosphatase conjugate was applied as the secondary antibody, with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium used for immunodetection.

RESULTS

Mitochondria from Etiolated Wheat Seedlings and Wheat Germ. Mitochondria prepared by the method of Day and Hanson (1977) from etiolated wheat seedlings had respiratory control ratios ranging from 5.0 to 7.0, although they deteriorated after an hour. ADP/O ratios were 2.7–2.9 using glutamate/malate as substrate, similar to those reported for maize mitochondria (Dutch et al., 1987). In contrast, the respiration rates of mitochondrial membrane fractions from wheat germ were not stimulated by ADP, and exogenously supplied cytochrome *c* had complete access to cytochrome oxidase, indicating that the mitochondria were not intact.

Mitochondrial membrane fractions isolated from etiolated wheat seedlings and from wheat germ appear to be very similar with respect to cytochrome content. The difference spectra (reduced minus oxidized) in Figure 1 show that the two sources of mitochondria yield similar amounts and ratios of cytochromes (0.12 nmol and 0.19 nmol aa_3 per milligram of mitochondrial protein for wheat germ and seedlings, respectively). The cytochrome oxidase specific activity of seedling mitochondria was usually higher (0.11 nmol of O_2 consumed per second per milligram protein), but only by about 10%. FPLC elution profiles of solubilized mitochondrial membranes from both sources were nearly identical, suggesting a similar protein complement. Wheat germ obtained from two different milling companies and at various times during the year yielded the same results.

Purification of Cytochrome Oxidase by FPLC. When mitochondrial membranes solubilized in lauryl maltoside were applied to a Pharmacia MonoQ column, oxidase eluted at 0.35–0.37 M KCl, allowing separation from the bc_1 complex, which elutes slightly later at 0.42–0.45 M KCl. An elution profile of solubilized wheat germ mitochondrial membranes is shown in Figure 2, and is nearly identical with that obtained from wheat seedling mitochondria (not shown). Concentration of the oxidase fractions by the inverted column method de-

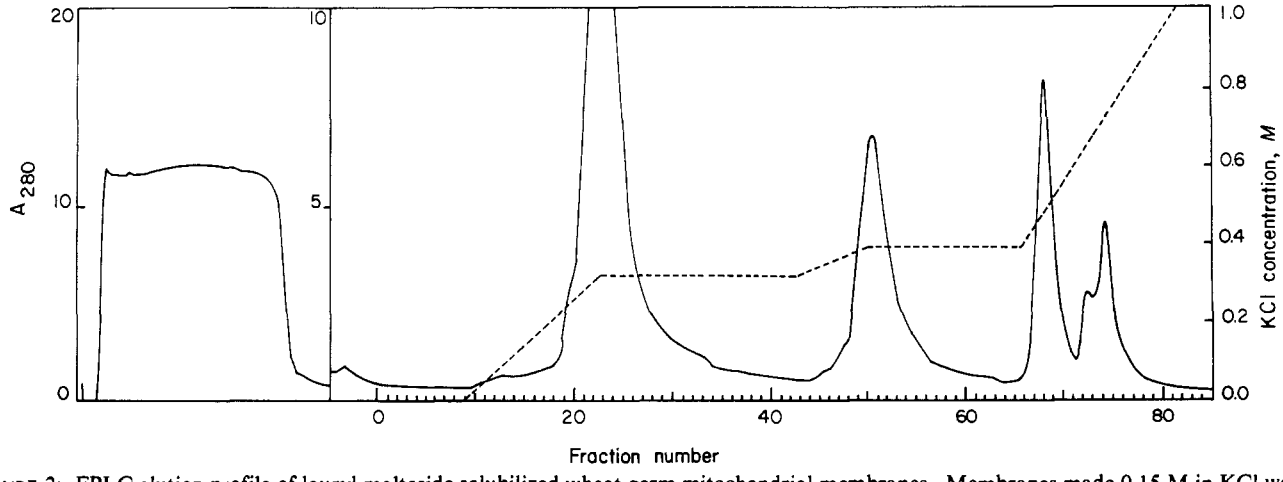

FIGURE 2: FPLC elution profile of lauryl maltoside solubilized wheat germ mitochondrial membranes. Membranes made 0.15 M in KCl were loaded onto a MonoQ 10/10 column and washed with FPLC buffer (5% sucrose, 20 mM KH_2PO_4, and 4 mM lauryl maltoside, pH 7.5) to remove unbound protein. Bound protein was eluted at 1.0 mL/min with a discontinuous KCl gradient: 0–0.32 M in 15 mL, 0.32 M for 20 mL, 0.32–0.39 M in 7 mL, 0.39 M for 15 mL, and 0.39–1.0 M in 16 mL. The solid line indicates the A_{280}, and the dashed line indicates the KCl gradient.

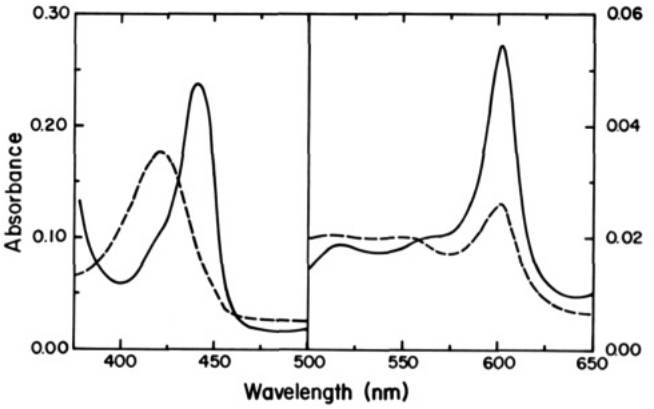

FIGURE 3: Visible spectrum of purified wheat germ cytochrome *c* oxidase after gel filtration on Superose 6. Oxidase was 2.5 μM in FPLC buffer (described in the legend of Figure 2). Air-oxidized (---); dithionite-reduced (—).

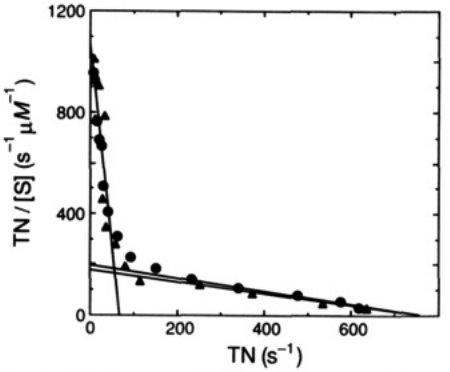

FIGURE 4: Eadie–Hofstee plot of the kinetics of reaction of solubilized mitochondrial membranes (●) and purified cytochrome *c* oxidase (▲) from wheat germ with horse heart cytochrome *c*. Assays were performed polarographically as described under Experimental Procedures. Oxidase concentration was 0.025 μM for membranes and 0.021 μM for purified oxidase; cytochrome *c* concentration ranged from 21.1 to 0.0084 μM.

scribed under Experimental Procedures can yield a highly concentrated enzyme; for example, 0.5 mL of 50 μM oxidase can be obtained from 900 mg of mitochondrial protein (in addition to more dilute fractions).

The visible spectra obtained from the final fraction are shown in Figure 3. Peaks at 441 and 602 nm in the reduced spectrum are blue-shifted relative to beef heart enzyme, whose reduced peaks are at 443 and 604 nm. A ratio of A_{280nm} to A_{420nm} of less than 2.2 is obtained after gel filtration. This final fraction has a turnover number of 500–800 s^{-1}, while fractions from earlier stages of purification have turnover numbers up to 1000 s^{-1}.

Reaction Kinetics with Cytochrome c. Both solubilized mitochondria and purified cytochrome oxidase from wheat germ exhibit biphasic kinetics similar to beef heart oxidase, as shown in Figure 4. The K_m of the initial high-affinity phase is 0.06 μM for both the purified and unpurified wheat germ oxidase; the K_m values of the second low-affinity phase are also not significantly different (4.2 and 3.8 μM, respectively). These results are in contrast to the kinetics observed for the maize oxidase whose purification was interrupted at several points by freezing and thawing; it exhibited a much lower affinity initial phase with a K_m value about 10-fold higher than that seen in the unpurified state (Ingle, unpublished data). These results emphasize the lability of the oxidase and the importance of a rapid purification scheme.

EPR Spectrum of Wheat Oxidase. The EPR spectra of the purified wheat germ oxidase as well as the purified maize

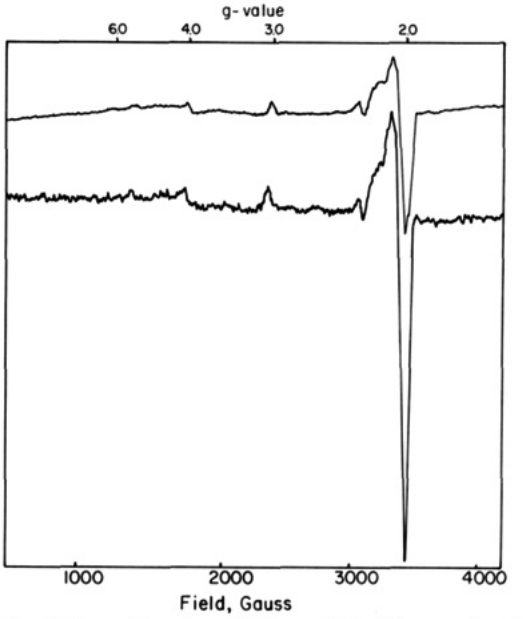

FIGURE 5: EPR spectra of purified corn (60 μM, upper trace) and wheat germ (64 μM, lower trace) cytochrome *c* oxidase in FPLC buffer (described in the legend of Figure 2) with 0.5 M KCl. Spectra were run at 10 K as described under Experimental Procedures.

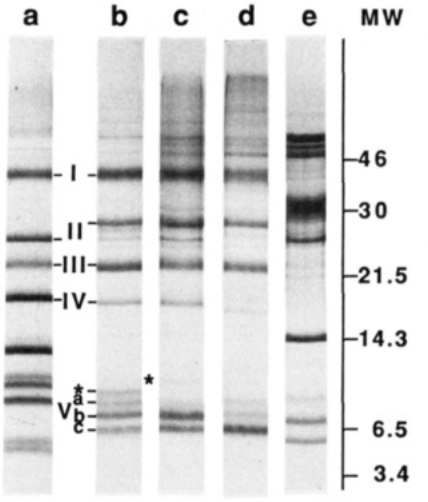

FIGURE 6: Subunit composition analysis of wheat germ and seedling cytochrome oxidase by SDS–PAGE. Lane a, beef heart oxidase, with subunit designations (I–IV) to the right; lane b, wheat germ oxidase concentrated from the first MonoQ run, with subunit designations to the left; lane c, wheat seedling oxidase concentrated from the first MonoQ run; the asterisk designates a possible alternative subunit as described under Results; lane d, corn seedling oxidase concentrated from the first MonoQ run fractions; lane e, bc_1 complex from the first MonoQ run. The positions of molecular weight ($\times 10^{-3}$) standards (Amersham rainbow markers) are shown to the right of lane e.

oxidase are shown in Figure 5. The $g = 2$ signals due to Cu_A are very similar and show the small copper nuclear hyperfine coupling typical of other cytochrome oxidases. The g_z signal at 3 and the g_y at 2.29 are normal for the low-spin iron of cytochrome *a*. The weak signal at $g = 4.3$ indicates very low levels of contaminating metals, such as nonintegral iron, and since there are no further major signals, cytochromes a_3 and Cu_B are EPR-silent in the resting enzyme. Thus, the EPR spectra demonstrate both the similarity of the wheat germ oxidase to other oxidases and also the purity and integrity of the enzyme.

Subunit Analysis of Wheat Oxidase. SDS–urea–polyacrylamide gels of wheat cytochrome oxidase (Figure 6) suggest a radically different subunit composition from the

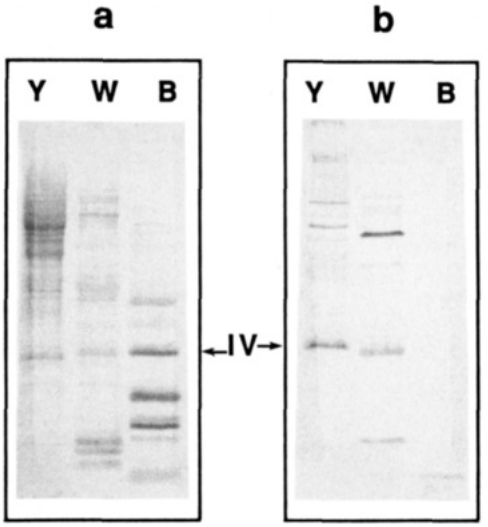

FIGURE 7: Western blot analysis of wheat germ cytochrome oxidase. Nitrocellulose blots in panels a and b contain lanes Y, yeast mitochondria; lanes W, wheat germ oxidase; and lanes B, beef heart oxidase. Panel a is the amido black stain of the nitrocellulose blot, and panel b is the immunodetection of subunit IV on the blot using monospecific polyclonal antibodies to yeast oxidase subunit IV. The position of subunit IV is indicated by the arrows.

mammalian enzyme, but a similar composition to what has been reported for other plant oxidases. Subunit designations of the beef heart enzyme are shown next to it in lane a. Lane b is wheat germ oxidase after MonoQ purification and concentration. The four largest bands have a similar migration behavior to the first four subunits of the beef heart enzyme in lane a. Lane e is the bc_1 complex as it appears after elution from the first MonoQ column. Comparison shows that contamination of the oxidase from bc_1 does exist (especially in the region below subunit II), though it is not spectrally evident in the fractions. Oxidases purified from wheat seedling and maize seedling mitochondria are shown in lanes c and d, respectively. The oxidase from wheat seedling has retained some subunit IV without the use of proteolytic inhibitors; however, corn seedling oxidase purified by a similar (but less rapid) procedure has little subunit IV except for some lower molecular weight bands that are perhaps degradation products.

Of particular interest is an additional band in the wheat germ cytochrome oxidase, labeled V*, that migrates near 9000 kDa immediately above the three small subunits labeled Va, -b, and -c. It is reproducibly present and has a different mobility than faint bands in that region of wheat seedling (lane c) or maize (lane d) oxidase. This band has not been observed in previous reports of purified plant oxidase. In the case of the seedling oxidases, a band at 10000 kDa is seen with variable intensity throughout the purification (lane d, asterisk); this may represent an alternative subunit.

Since subunit IV was readily lost during purification, it was important to determine whether it was an authentic oxidase subunit. The availability of monospecific polyclonal antibodies to yeast oxidase subunit IV allowed us to test whether it was a related peptide. Nitrocellulose blots of yeast mitochondria (Y) and purified beef heart (B) and wheat germ (W) oxidases are shown in Figure 7. Panel a is the amido black stain of these transfers; all the subunits of the oxidases are visible, although subunits I and III transfer less efficiently. Panel b shows the same samples as in (a) after incubation with monospecific polyclonal antibodies to yeast oxidase subunit IV, followed by secondary antibody labeling and alkaline phosphatase color development. Subunit IV of the yeast oxidase and wheat germ oxidase react with these antibodies, but the purified beef heart oxidase does not. The position of subunit IV, which is higher in the yeast than in the beef heart or wheat germ oxidases, is marked by the arrows. Some nonspecific reaction with higher molecular weight species is also seen.

Discussion

Commercial wheat germ is inexpensive, readily available, and not highly variable. If stored cold (4 °C) in closed containers, it can be used for at least 6 months. Although intact mitochondria were not obtained, commercial wheat germ yields a mitochondrial membrane fraction with a similar content of cytochromes bc_1 and aa_3 to that found in mitochondria isolated from etiolated seedlings. The relatively easy and rapid preparation we have developed offers the potential of isolating large quantities of cytochrome oxidase and many other plant mitochondrial proteins as well. Since the germ is an embryonic tissue, developmental and tissue-specific changes in enzymes may also be studied.

Cytochrome *c* oxidase can be purified in relatively large amounts from these wheat germ mitochondrial membranes using anion-exchange FPLC. Thus, we can now do studies on plant oxidase that were formerly not possible due to lack of material, such as protein sequencing and Raman spectroscopy [see de Paula et al. (1990)]. In addition to quantity, our enzyme is highly active and displays biphasic kinetics similar to beef heart oxidase. Previous studies on plant cytochrome oxidases show significant loss of activity during purification but do not provide any analysis of the enzyme's kinetic characteristics. The lability of the enzyme is also illustrated by our studies on maize oxidase purified by a similar but less rapid procedure: a dramatic loss of affinity for cytochrome *c* in the initial kinetic phase is observed, compared to the membrane-bound form. Concomitantly, the maize oxidase shows almost complete loss of subunit IV, which suggests a possible role for this subunit in determining the cytochrome *c* affinity of the initial kinetic phase, though other less obvious proteolytic damage could be involved. Subunit IV appears to be particularly labile to proteolytic degradation; even when proteolytic inhibitors are used in the purification of wheat germ oxidase, subunit IV is present in less than stoichiometric amounts. In general, rapid purification appears to be essential for maintaining the native characteristics of plant cytochrome oxidase.

The spectral properties of the wheat germ and seedling oxidases are very similar to those reported for maize and other plant oxidases. The blue shift of the reduced peaks indicates an altered heme *a* environment (Chance et al., 1968), a conclusion that is supported by analysis of the plant oxidase by resonance Raman spectroscopy (de Paula et al., 1990). The similarity of the $g = 2$ EPR signal of wheat and corn cytochrome oxidases suggests that Cu_A has the same configuration in both enzymes. Thus, it is unlikely that an amino acid substitution has taken place at the Cu_A site. This result supports the evidence that mRNA editing has occurred in the codon for residue 228 of subunit II to restore the conserved cysteine at this site (Covello & Gray, 1989). Amino acid sequence information from wheat oxidase subunit II is necessary to confirm this observation.

We have not yet demonstrated the enzyme's proton pumping capabilities, but the normal ADP/O ratios measured for intact wheat seedling mitochondria suggest that it does pump protons. Furthermore, purified enzyme reconstituted into asolectin vesicles has good respiratory control ratios (5–6, data not shown). Since the EPR indicates a normal Cu_A, wheat oxidase does not challenge the theory of Chan regarding a central role of this metal in proton pumping.

Plant oxidases have previously been shown to have nuclear-encoded subunits that differ from animal or yeast oxidases. The larger subunits (I, II, III, IV) of the wheat germ oxidase appear to be similar in size and antigenicity to the yeast enzyme, but the three or four smaller subunits below 10000 kDa are similar only to other plant oxidases. A subunit of about 9000 kDa is retained during purification of the wheat germ oxidase, but is not seen at any stage of purification of the wheat seedling enzyme, whereas the latter enzyme appears to have a larger (10000 kDa) peptide that does not appear in the germ. This suggests a possible developmental form of the oxidase in wheat, similar to the developmental forms found in slime mold (Bisson & Schiavo, 1986). Further work is needed, however, to verify the true subunit composition of the wheat complex.

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