Changes in the Mitochondrial Proteome during the Anoxia to Air Transition in Rice Focus around Cytochrome-containing Respiratory Complexes*

A. Harvey Millar†, Alice E. Trend, and Joshua L. Heazlewood

From the Plant Molecular Biology Group, School of Biomedical and Chemical Sciences, the University of Western Australia, Crawley 6009, Western Australia, Australia

The ability of rice seedlings to grow from dry seed under anoxia provides a rare opportunity in a multicellular eukaryote to study the stages of mitochondrial biogenesis triggered by oxygen availability. The function and proteome of rice mitochondria synthesized under 6 days of anoxia following 1 day of air adaptation have been compared with mitochondria isolated from 7-day aerobically grown rice seedlings. Rice coleoptiles grown under anoxia, and the mitochondria isolated from them respired very slowly compared with air-adapted and air-grown seedlings. Immunodetection of key mitochondrial protein markers, isoelectric focusing electrophoresis followed by SDS-PAGE to make soluble mitochondrial proteome maps, and shotgun sequencing of mitochondrial proteins by liquid chromatography-tandem mass spectrometry all revealed similar patterns of the major function categories of mitochondrial proteins from both anoxic and air-adapted samples. Activity analysis showed respiratory oxidases markedly increased in activity during the air adaptation of seedlings. Blue-native electrophoresis followed by SDS-PAGE of mitochondrial membrane proteins clearly showed the very low abundance of assembled bc1 complex and cytochrome c oxidase complex in the mitochondrial membrane in anoxic samples and the dramatic increase in the abundance of these complexes on air adaptation. Total heme content, cytochrome absorbance spectra, and the electron carrier, cytochrome c, also increased markedly on air adaptation. These results likely reflect limited heme synthesis for cytochrome assembly in the absence of oxygen and represent a discrete and reversible blockage of full mitochondrial biogenesis in this anoxia-tolerant species.

The structure and functional status of mitochondria in the absence of O2 has intrigued researchers for decades. Early studies in yeast initially suggested that mitochondria degraded and were lost during anaerobic growth (1), but later reduced mitochondrial structures with altered morphology and those lacking respiratory activity were clearly identified in anaerobic yeast and termed “protomitochondria” (2). Extending these studies to animals has been hampered by the susceptibility of many cells to long term lowered oxygen concentrations, which simply lead to energy deprivation and cell death (3, 4). Many experiments have been conducted on tissues that develop in the presence of O2 and are assessed during short term oxygen deprivation. Such studies have shown that mammalian mitochondria may function as oxygen sensors and secondary messengers by increasing their generation of reactive oxygen species during the early stages of oxygen deprivation (5). Induction of some “hypoxic nuclear genes” in both mammals and yeast requires mitochondrial respiration and cytochrome c oxidase has been shown to function as a key oxygen sensor during this process (6).

Within the plant kingdom a very broad plasticity in primary metabolic function has developed to cope with altered oxygen availability (7, 8). Some plant species exhibit extreme tolerance to prolonged anoxic conditions by maintaining an energy charge through increased glycolysis and redistributed energy allocation (9). Rice is one of the most anoxia-tolerant plant crop species (10), is able to germinate and sustain early seedling growth in strictly anoxic solutions (11, 12) or even under a high vacuum (13), and can readily return to atmospheric or aerobic conditions without substantial damage (14, 15). Under field conditions rice is often grown in anaerobic rice field conditions rice to meet aerobic conditions as it grows upward (16).

On the return of anoxically grown rice seedlings to air there are a number of well documented responses to sudden oxygen availability including increased respiratory rate (14), elevation of antioxidant enzymes such as catalase, ascorbate peroxidase, superoxide dismutase, dehydroascorbate/monohydroascorbate reductase, and glutathione reductase (15, 17, 18), and heightened levels of small molecule antioxidants such as glutathione, ascorbate, and α-tocopherol (15, 17). Mitochondrial structures appear to proliferate in rice seedlings even when they are grown under anoxic conditions from dry seed. Ultrastructural studies of mitochondria from anoxic rice tissues show a matrix of lower density and a more defined membrane cristae structure than is apparent in mitochondria from aerobic seedlings (19, 20). Couee et al. (19) have also shown that rice mitochondria from anoxic tissues can carry out in organello protein synthesis when the functional respiratory chain is inhibited. This suggests rice mitochondria grown in anoxic conditions can bypass the electron transport function requirement for organelar protein synthesis that is apparent in most plant mitochondria. Fox and Kennedy (21) found that the activities of the tricarboxylic acid cycle enzymes succinyl-CoA synthase and citrate synthase were similar in aerobically and anaerobically grown seedlings, whereas the activities of 2-oxoglutarate dehy-

* This work was supported by grants from the Australian Research Council through the Discovery Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† An Australian Research Council QEII Research Fellow. To whom correspondence should be addressed: Biochemistry and Molecular Biology, School of Biomedical and Chemical Sciences, Faculty of Life and Physical Sciences, The University of Western Australia, Crawley, WA 6009, W. A., Australia. Tel.: 61-8-93807245; Fax: 61-8-93801148; E-mail: hmillar@cyllene.uwa.edu.au.

This paper is available on line at http://www.jbc.org

Received for publication, June 1, 2004, and in revised form, July 7, 2004
Published, JBC Papers in Press, July 16, 2004, DOI 10.1074/jbc.M406015200
Mitochondria in Anoxic to Aerobic Transition in Rice

drogenase complex, aconitate, isocitrate dehydrogenase, and fumarase were reduced in anaerobic seedlings. The rate of succinate oxidation and succinate dehydrogenase activity has also been reported to be lower in mitochondria from rice seedlings grown submerged but increased during adaptation to air. Cytochromes have been shown to be present in anoxically grown rice but at lower levels than in aerobic controls (12), and the mitochondrial cytochrome content increases rapidly on air adaptation from anoxia (22). Reduced cytochrome gene expression has been reported to respond to oxygen deprivation and air adaptation in rice (23). Transcript abundance from nuclear-encoded genes for components of both the cytochrome and alternative oxidases in mitochondria were reduced during anoxia and induced by air adaptation. The same authors have shown recently a specific isofrm of mitochondrial aldehyde dehydrogenase (ALDH2a) is induced on re-aeration of hypoxic rice seedlings, putatively to defend against damage from acetaldehyde accumulation post-submergence (24).

The degree to which these reported changes in specific mitochondrial activities and transcript levels explain mitochondrial dysfunction from anoxically grown rice seedlings, and to what extent dysfunction is because of transcriptional, translational, or post-translational factors remains unclear. Further, the much reduced state of mitochondria in anoxia in yeast suggest an oxygen-derived trigger for full mitochondrial biogenesis, but the nature of mitochondrial proteomes under anoxia have not been systematically investigated to date in either anoxia-tolerant or -intolerant plants. We have recently studied the rice mitochondrial proteome in air-grown seedlings (25). By following the induction of respiration in rice coleoptiles during air adaptation of anoxic seedlings and assessing the changes in the rice mitochondrial proteome during this transition, we showed that much of the mitochondrial proteome appears relatively unaffected by anaerobic growth. However, we showed that the specific low abundance of cytochrome-containing complexes of the respiratory chain likely explains the markedly reduced respiratory function of both intact anoxically grown rice seedlings and the mitochondria they contain.

EXPERIMENTAL PROCEDURES

Plant Material—Rice (Oryza sativa L. cv. Amraroo) seeds were germinated in 5-liter conical flasks containing 2.5 liters of growth medium (0.5 mM MES, 0.5 mM CaCl2, 6 mM/gliter carbenicillin, pH 6.5). The seeds were sterilized using 70% ethanol and 5% (v/v) bleach and washed before being placed in conical flasks. The flasks were incubated at 25°C in the dark. Depending on the treatment required, flasks were continuously bubbled with either commercial grade N2 or air at 1 liter min−1 using spargers placed below the water level. The presence of O2 was monitored using Anaerobic Indicator™ (Don Whitley Scientific Ltd.) according to manufacturer’s instructions.

Mitochondrial Isolation—The coleoptiles from ~16,000 seedlings were separated from their seeds in a large container of water using a manual rubbering technique. Coleoptiles float, and seeds sink following separation. The floating coleoptiles were skimmed from the surface using netting, weighed, and placed on ice. This operation could be performed over a self-cleaning 26% (w/v) Percoll (Amersham Biosciences) gradient made in wash buffer and centrifuged at 35,000 × g for 45 min. The mitochondria formed a band toward the bottom of the tube. Aspirated mitochondria samples were concentrated, and Percoll was removed by centrifugation twice in wash buffer for 15 min at 27,200 × g.

Oxygen Consumption—Whole tissue O2 consumption rates were recorded using 5–10 coleoptiles, seeds, or whole seedlings in 3 ml of fresh rice growth medium in a Clarke-type oxygen electrode (Rank Brothers, Cambridge). KCN (1 mM) and salicylhydroxamic acid (1 mM) were used to inhibit respiratory oxidases, showing that oxygen consumption recorded from whole tissues was >90% respiratory in origin. O2 consumption rate in these isolated mitochondria was measured in the presence of 10 mM succinate and pyruvate (1 mM) in 1 ml of reaction medium (0.5 mM mannitol, 10 mM TES-KOH, pH 7.5, 5 mM KH2PO4, 10 mM NaCl, 2 mM MgSO4, and 0.1% (w/v) bovine serum albumin). No respiratory rates from mitochondrial samples were detected prior to substrate additions. The following reagents and inhibitors were added to the mitochondria to examine mitochondrial integrity and function, ascorbate (10 mM), cytochrome c (25 μM), Triton X-100 (0.05% w/v), KCN (1 mM), ATP (100 μM), NADH (1 mM), ADP (0.05 mM), myxothiazol (5 μM), pyruvate (5 mM), dithiothreitol (2 mM), n-propylgallate (5 μM), salicylhydroxamic acid (1 mM), KCN (1 mM), malate (10 mM), NAD+ (1 mM), and thiamine pyrophosphate (1 mM). Succinate-dependent respiration was measured in the presence of ATP to activate succinate dehydrogenase in the presence of dehydrogenase oxidase to inhibit respiratory rate. Succinate- and NADH-dependent respiration was measured in the same manner with these dual substrates. Malate and pyruvate-dependent respiration was measured with the addition of NAD and thiamine pyrophosphate as cofactors and ADP to maximize respiratory rate. The alternative oxidase respiratory rate was measured in the presence of succinate and NADH to maximize electron flux, myxothiazol and KCN to block cytochrome pathway operation, and pyruvate and dithiothreitol to fully activate alternative oxidase. The alternative oxidase rate was fully inhibited by the addition of the inhibitor, n-propylgallate. Cytochrome c oxidase activity was measured by the solubilization of mitochondrial membranes with Triton X-100 and the determination of cytochrome c oxidase activity using a system consisting of exogenous cytochrome c and ascorbate. The cytochrome c-dependent oxygen consumption rate was inhibited by KCN.

Thiobarbituric Acid Reactive Substances, Heme, and Cytochrome Assays—The measurement of malondialdehyde equivalents was based on the method of Hodges et al. (26). The total heme content of mitochondrial samples was measured as pyridine hemochrome after a reaction of heme with an alkaline pyridine solution (0.2 mM NaOH, 4.2 mM pyridine) according to Appleby and Bergersen (27). Hemochrom content was assessed from reduced-minus-oxidized spectra (A550–650) using trace addition of sodium dithionite or potassium ferricyanide to reduce or oxidize, respectively. A550–650 from these spectra and the extinction coefficient ε550=22.4 mmol−1 cm−1 were used to determine the heme content according to established formulas (27). Cytochrome reduced-minus-oxidized spectra (A420–600) were recorded in mitochondrial samples suspended in wash buffer (2 mg of mitochondrial protein ml−1). Succinate (10 mM), NADH (0.1 mM), and sodium dithionite were added to reduce cytochromes, and no treatment was used as the control oxidized cytochrome sample. Peak identifications were undertaken by comparison to work of Chance and Williams (28) and Shibasaka and Tsujii (22).

Western Blotting and Immunodetection—Mitochondrial proteins (50 μg) were separated using one-dimensional SDS-PAGE, transferred to nitrocellulose membrane, blocked and analyzed with a series of antibodies directed to tricarboxylic acid cycle components according to standard protocols. Lipidic acid attached to acrylamides in 2-oxoglutarate and pyruvate dehydrogenase complexes and H protein of glycine decarboxylase were identified with the polyclonal antibody raised to lipidic acid (29). The Ela1 subunit of pyruvate dehydrogenase complex, alternative oxidase, β-subunit of ATP-synthase, HSP70, and outer mitochondrial membrane voltage-dependent anion channel mitochondrial antibodies raised against maize mitochondrial proteins from Dr. Tom Elthon, University of Nebraska, Lincoln, NE. Cytochrome c was a monoclonal raised to the pigeon enzyme (Pharmpineing). A chemiluminescence detection kit was used to visualize the immunoreaction that was then recorded using an Image Analyzer (LAS 100, Fuji, Tokyo). Densitometrically determined, chemiluminescence was quantified with a Image Gauge version 3 software (Fuji, Tokyo).

Two-dimensional Gel Separation and Staining—Isoelectric focusing/SDS-PAGE analysis was carried out according to Millar et al. (35) using non-linear 3–10 pH IEF strips. Blue-native PAGE analysis was carried
Mitochondria in Anoxic to Aerobic Transition in Rice

Fig. 1. Rice coleoptile growth and lipid peroxidation during anoxic and aerobic growth. Coleoptiles from 7-day anoxic growth plants (white), after 6 days of anoxic growth and 1 day of aerobic adaptation (gray), or after 7 days of growth in aerobic conditions (black) are shown. A, coleoptile length in mm (mean ± S.E. (n = 30)); B, coleoptile mass in mg of FW (mean ± S.E. (n = 20)); C, lipid peroxidation as malondialdehyde (MDA) equivalents as nmol/g of FW (mean ± S.E. (n = 6)).

out according to published methods (30), and second dimension SDS-PAGE was done by Tris-Tricine separations. Protein spots from polyacrylamide gels were excised and prepared for analysis by MS according to Sweetlove et al. (31). Proteins were visualized by colloidal Coomassie Brilliant Blue G250 staining; gels were placed in a solution of 17% (w/v) ammonium sul fate, 34% (v/v) methanol, 3% (w/v) phosphoric acid, 0.1% (w/v) Coomassie Brilliant Blue G250 for 10 h and destained in 0.5% (v/v) phosphoric acid for 24 h. Gels were scanned using a 12-bit transparency scanner (Image Scanner, Amersham Biosciences). Changes in spot intensity were analyzed by ImageMaster two-dimensional Elite software (Amersham Biosciences) on 12-bit TIFF images using background subtraction by the lowest-on-boundary method. Fold changes in spot volumes between treatments were calculated and are presented in Table I.

Mass Spectrometry—For BN-PAGE samples, identification was carried out as indicated in Heazlewood et al. (25). For LC-MS/MS, the mitochondrial samples of 100-μg were acetone-precipitated at −20 °C overnight. The resulting precipitated protein pellet was resuspended in 100 mM Tris-HCl (pH 8.5). The protein lysate was digested overnight at 37 °C with trypsin 1/10 (w/w), and insoluble material was removed by centrifugation. Digested samples of 15–20-μg were analyzed on a QStar Pulsar MS/MS system (Applied Biosystems) utilizing an inline Agilent 1100 capillary LC system incorporating a Zorbax C18 reverse phase column (Agilent) for peptide separations. Peptides were analyzed by MS over an 8-h elution period with increasing acetonitrile concentrations from 2 to 80% (v/v) in H2O and 0.1% (v/v) formic acid. Ions were automatically selected for the N2 collision cell by the Analyst QS software package (Applied Biosystems). Data from both gel-extracted peptides and whole mitochondrial lysates were analyzed by an in-house data base comprising TIGR and NCBI rice protein sets, which were searched with the resulting MS/MS data at error tolerances of MS ± 0.15 and MS/MS ± 0.05 using ProID, or analyzed online using the Mascot data base2 with MS/MS data at error tolerances of MS ± 1.2 and MS/MS ± 0.6.

RESULTS

Rice Seedling Growth and Respiratory Capacity under Anoxia and in Air—Over the course of 7 days, cv. Amaroo rice seeds germinated, and coleoptiles elongated under strict anoxia. These seedlings did not exhibit any root elongation or leaf development, consistent with the long known requirement of trace levels of O2 for these developmental processes to initiate (32). In addition, chemical colorimetric indicators of anaerobiosis showed that O2 levels were negligible during the period of growth and did not fluctuate during the experiment (data not shown). After 7 days, coleoptiles had emerged and elongated to −20 mm in length. The Amaroo cultivar has been shown to be highly tolerant to anoxia in comparison to other commercial rice cultivars (33). The transfer of coleoptiles on day 6 to sparging with air for 24 h resulted in only a small increase in coleoptile length but no significant change in mass by day 7, compared with the 7-day anoxic controls (Fig. 1, A and B). Root initiation was observed during the 24 h of air adaptation, and aeration of the solution was also indicated by colorimetric indicators of anaerobiosis (data not shown). In comparison, the germination of rice seedlings in air-saturated solutions for 7 days yielded seedlings with 70-mm long coleoptiles with nearly double the fresh weight mass of anoxic controls (Fig. 1, A and B), and this growth was accompanied with extensive root and primary leaf development.

The assessment of O2 exposure and the degree of O2 toxicity following adaptation was also determined by measuring lipid peroxidation levels as malondialdehyde equivalents in the rice coleoptile tissue. This analysis revealed that the exposure of rice to air for 24 h more than doubled the extractable malondialdehyde content, whereas germination in air for the whole 7-day period yielded malondialdehyde levels more than 6 times those found in anoxic controls (Fig. 1C).

A portion of the seedlings from each of the three treatment groups was rapidly removed, and O2 consumption capacities in air-saturated conditions were assessed (Fig. 2A). Although all seedlings were capable of O2 consumption, seedlings from the 24-h air-adapted and the 7-day air-grown treatments exhibited nearly twice the respiratory rate of anoxic control seedlings on a FW basis. When the O2 consumption capacity of the seed and coleoptiles was assessed separately it was evident that nearly all of the increased O2 consumption capacity of the 24-h air-treated seedlings was due to anoxic controls was because of coleoptile respiration. On the other hand, in 7-day air-grown seedlings both the seed and the coleoptile contributed to the high respiratory rate. The addition of 50 mM glucose and/or the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (10 μM) to coleoptiles did not increase respiratory rates, whereas more than 90% of coleoptile rates were inhibited by addition of 1 mM KCN and 1 mM salicyldihydroxamic acid (data not shown).

Respiration by Mitochondria from Anoxically and Air-grown Rice Coleoptiles—Mitochondria were isolated from coleoptiles grown under the three treatments regimes. The pattern of maximum O2 consumption rates between treatments was similar using succinate/ADP or a combination of succinate/NADH/ADP as substrates (Fig. 2B). Mitochondria from anoxic treatments exhibited a 3–4-fold lower O2 consumption rate than mitochondria from the 24-h air-adapted seedlings. The mitochondria from the air-adapted and air-grown seedlings were not significantly different. Malate- and pyruvate-dependent O2 consumption was lower than what was measured during succinate and NADH oxidation in all samples; it was nearly 8-fold lower in anoxically grown samples than in air-adapted samples on a mitochondrial protein basis. All respiration rates were largely inhibited by the cytochrome pathway inhibitors KCN and myxothiazol, except in the case of the 24-h air-adapted
samples that consistently revealed a KCN-insensitive respiratory rate that could be inhibited by the alternative oxidase inhibitor n-propylgallate. A measurement of the cytochrome c oxidase (COX) activity revealed a very large difference between anoxically grown and air-adapted samples. The 24 h of air adaptation appeared to be sufficient to elevate COX activity close to the levels measured in mitochondria from air-grown seedlings (Fig. 2B). Together these data suggested that respiratory capacity in mitochondria from anoxically grown coleoptiles was greatly limited by COX activity, with the respiratory rates driven by succinate and NADH oxidation approaching the apparent COX capacity.

Western blot analysis of selected mitochondrial proteins revealed that although there were some changes in abundance of specific components between treatments, overall a maintenance of key mitochondrial proteins in all three treatments was observed (Fig. 3). The outer membrane marker VDAC, the inner membrane marker β-subunit ATP synthase, the matrix markers of pyruvate dehydrogenase complex subunits and HSP70, were all unchanged by the treatments on a mitochondrial protein basis. The alternative oxidase was not detectable in anoxically grown or air-grown coleoptiles but was clearly induced by a 24-h air adaptation of anoxic coleoptiles. This was consistent with the KCN-insensitive O₂ consumption recorded in these samples (Fig. 2B). The H protein of glycine decarboxylase was not present in anoxic treatments but was found to increase with the degree of air exposure, consistent with the onset of leaf development.

Two-dimensional gel blot analysis of selected proteins—To further compare the protein complement between the anoxic samples and the near fully functioning 24-h air-adapted mitochondrial samples, soluble proteins from these samples were separated by classical IEF/SDS-PAGE. These separations of 1 mg of mitochondrial protein revealed very similar protein spot patterns between the two treatments. Typical examples of a paired experiment are shown in Fig. 4. Both two-dimensional gel patterns showed great consistency with the published rice shoot mitochondrial proteome analysis (25). The apparently changing pattern of a series of protein spots highlighted in Fig. 4 are simply differences in the amount of bovine serum albumin in samples used in the mitochondrial isolation procedure. Thus the main soluble mitochondrial proteome apparent on IEF/SDS-PAGE appears to be synthesized and maintained under anoxia and was little altered by air adaptation.

Non-gel LC-MS/MS assessment of mitochondrial contents from anoxic and 24-h air-treated coleoptiles—To broadly identify the components of the mitochondria proteomes from the anoxic and 24-h air-adapted coleoptiles, trypsin-digested protein samples were directly analyzed by tandem mass spectrometry to identify the major proteins present in each case. We have successfully used this method in both rice and Arabidopsis previously to define mitochondrial proteomes (25, 34). Peptides from both samples were randomly sequenced during an 8-h elution from a C18 reverse-phase column with a gradient of 2–80% (v/v) acetonitrile. The number of peptides identified from proteins of particular functional classes are displayed in Fig. 5. During the 24 h of air adaptation there was an increasing proportion of proteins in chaperones and mitochondrial carbon metabolism categories that were identified, but generally, the profile of the mitochondrial functional categories present was similar under both anoxia and air adaptation and broadly reflects the content of mitochondria we have previously observed in rice and Arabidopsis (34, 35).

A detailed inspection of the contents in the respiratory chain category in Fig. 5 revealed that there were no reported peptides for either the b/c₁ complex (complex III) or the cytochrome c oxidase (complex IV) in anoxic samples, but ATP synthase (complex V), NADH:UQ oxidoreductase (complex I), and succinate dehydrogenase (complex II) components were found in
both anoxic and air-adapted samples (data not shown). Therefore, it appeared that rather than a general increased biogenesis of large parts of the mitochondrial proteome occurring, it was more likely that very specific proteins required for mitochondrial function, notably respiratory chain components, may be induced to facilitate the rapid functional engagement of mitochondria in O₂ consumption during air adaptation of rice seedlings.

**BN-polyacrylamide Gels of Electron Transport Chain Complexes**—Many electron transport chain components are not apparent on IEF-SDS/PAGE gel separations because of the hydrophobicity of the proteins (25). So to further investigate possible differences in electron transport chain complexes, Blue-native gels were used to separate membrane protein complexes, and then SDS-PAGE was used to separate the components of these complexes. Recently, Eubel et al. (36) used a series of non-ionic detergent concentrations to develop BN-PAGE gels that display complexes I, II, III, IV, and V of the respiratory chain as discrete complexes from plant mitochondrial samples. Using digitonin as the non-ionic detergent and the method of Eubel et al. (36) we could successfully separate anoxic and air-adapted mitochondrial membrane samples (Fig. 6). Complexes I and V (F₁F₀-ATP synthase) were identified by protein component sizes, locations, and comparison to published reports (25, 36). These complexes were present in both treatments with little apparent difference in relative abundance. Densitometry analysis of six protein bands in each of these complexes showed a range of 0.7–1.3-fold change during air adaptation for these subunits (Table I). Several other lower
molecular mass complexes were the most apparent in the air-adapted samples. The proteins in these complexes (Fig. 6, spots 1–12) were either only faintly visible in anoxic samples or appeared to be absent (Fig. 6B). Densitometry showed that these spots were typically 3–7-fold higher in intensity above the gel background in normalized comparisons between anoxic and air-adapted samples gels. This indicated a 3–7-fold increase in protein spot abundances by the 1-day period of air adaptation (Table I). Excision, in-gel protein digestion, and tandem mass spectrometry of 12 spots from the air-adapted gel revealed that the complex containing spots 1–6 was the b/c₁ complex, whereas the spots 7–12 were all subunits of the cytochrome c oxidase complex (Table I). The two presentations of the b/c₁ complex either side of the F₉F₁-ATP synthase upon air adaptation (Fig. 6) likely reflects the presence of supercomplexes in the digitonin extraction, the smaller one being a dimer of complex III and the larger being complex IV attached to dimers of complex III (37). This interpretation is consistent with the presence of COXII (Fig. 6, spot 11) and COXIII (spot 12) as bands in the larger form of complex III. Six spots (Fig. 6B, 13–18) from the gel of anoxic samples were analyzed, because they were in similar positions to the b/c₁ and cytochrome c oxidase complexes identified by spots 1–12. Spots 13 and 15 we directly identified as subunits of the b/c₁ complex but were significantly lower in abundance than those proteins in spots 1–6. Spots 16 and 18 did not contain peptides that matched cytochrome c oxidase, instead these represent aldolase and the d subunit of the ATP synthase (Table I). Spectra obtained from peptides from spots 14 and 17 were of low intensity and poor quality and did not match predicted protein sequences.

**Heme and Cytochrome Content of Mitochondria from Anoxic and Air-adapted Samples**—The rice mitochondria isolated from anoxic seedlings were visibly gray, whereas air-grown or air-adapted mitochondria have the red-brown hue expected for heme-rich organelles. To quantify these differences, spectrophotometric measurements of cytochrome absorbances and total heme content were undertaken (Fig. 7). Room temperature reduced-minus-oxidized spectra (400–700 nm) showed the expected absorbance maxima for the a and γ peaks of a and c cytochromes. The b cytochrome absorbance peaks are obscured in the a and b cytochrome peaks in the room temperature spectra (28). The absorbance maxima in the 2 mg of protein ml⁻¹ spectra indicated a 4–8-fold higher cytochrome content in the mitochondria from air-adapted samples compared with anoxically grown samples (Fig. 7A). These differences are consistent with the much lower levels of complexes III and IV in the BN-PAGE gels from anoxically grown samples (Fig. 6). To determine whether this lack of reducible cytochromes was because of a general lack of heme in the mitochondria samples, alkaline pyridine was used to react with heme to form pyridine hemochromes, which was then quantified by spectrometry (Fig. 7B). This showed a 5-fold increase in total heme content of mitochondria during air adaptation of rice coleoptiles. The abundance of cytochrome c that acts as an electron shuttle between complexes III and IV was also analyzed directly by antibodies raised to pigeon cytochrome c. Cytochrome c protein abundance was found, based on densitometry measurements, to increase more than 7-fold during air adaptation (Fig. 7C).

**DISCUSSION**

The existence of mitochondrial structures that require specific biogenesis triggers to initiate a pathway to maturation and proliferation has been documented in a variety of eukaryotes (38–40). Reduced mitochondrial structures have been observed in both dried seeds and in meristematic zones in a variety of plants (41–43). Anaerobiosis is another scenario where such protomitochondria have been proposed to exist in both plants and yeast (2, 6, 12, 19, 23). Plant studies, like those in many animals, have been hampered by the lack of extended anoxia tolerance of cells to truly observe mitochondria that have been formed in the absence of oxygen. Rice offers the rare ability to conduct experiments on plant tissues grown from dry seed in anoxia. In our work, mitochondria from rice coleoptiles were in a very low competence state under anoxia but were not in a greatly reduced state in terms of their wider proteome complement. Adaptation to air rapidly facilitates the synthesis and/or assembly of the deficient components to yield mitochondria within 24 h that are not dramatically different from mitochondria of aerobically grown plants.

Cytochromes have been shown previously to be lower in abundance in anoxic rice samples than in air-grown samples (12, 22). Mitochondrial cytochromes incorporate heme groups that are synthesized in a complex set of tetrapyrrrole biosynthesis reactions in plants involving enzymes in the cytosol and

| Spot No. | Description | GI number | Accession | MP | Cov | MOWSE | MM gel | MM match | Fold increase by 1-day O₂ |
|----------|-------------|-----------|-----------|----|-----|-------|--------|----------|------------------------|
| 1, 13 MPP | — | 34906418 | NP_914556 | 12 | 19 | 555 | 60 | 90.2 | 0.7–1.3 |
| Cytochrome b | — | 27808645 | P14583 | 3 | 6 | 117 | 36 | 44.9 | 4.8–5.4 |
| Cytochrome c₁ | — | 34907202 | NP_914948 | 4 | 17 | 180 | 28 | 33.2 | 3.3–4.9 |
| REISEKE Fc-S | 92489052 | CAE05161 | 10 | 40 | 492 | 19 | 30.1 | 3.7–4.9 |
| UCR 14 kDa | 30103022 | AAP21435 | 5 | 34 | 288 | 12 | 15 | 3.3–6.4 |
| UCR 8 kDa | 34898456 | NP_910574 | 1 | 16 | 36 | 8 | 8.4 | 4.3–6.3 |
| COX I | 11690303 | P14578 | 1 | 1 | 35 | 40 | 57.7 | 3.9–4.4 |
| COX II | 117032 | P04373 | 3 | 10 | 114 | 30 | 29.2 | 3.1–3.2 |
| COX Vb | 34912592 | NP_917643 | 4 | 25 | 164 | 24 | 18 | 3.5–3.7 |
| COX Vla | 34897018 | NP_909855 | 1 | 7 | 59 | 10 | 10.7 | 5.4–7.7 |
| COX II | 11690303 | P14578 | 1 | 1 | 37 | 40 | 57.7 | 5.0–6.0 |
| COX II | 117032 | P04373 | 2 | 8 | 106 | 30 | 29.2 | 5.0–7.5 |
| Aldolase | 7436806 | S65073 | 14 | 35 | 484 | 40 | 39 | — |
| ATP synthase d | 32352148 | BAC78567 | 3 | 13 | 67 | 22 | 20 | — |
the mitochondria. Plants also synthesize the related tetrapyrrole, chlorophyll, and other plastidic cytochromes in a parallel pathway operating in plastids (44). The last common step to heme and chlorophyll is the oxidation of protoporphyrinogen IX to protoporphyrin IX in a six-electron oxidation step that uses O₂ as an electron acceptor. Isoforms of the protoporphyrinogen IX oxidase are present in both mitochondria and chloroplasts in plants (45). In the absence of oxygen, this reaction will be halted, resulting in a severe limitation in the heme available to the plant for cytochrome assembly. In yeast, mutations in heme synthesis lead to a lack of assembly of heme containing respiratory complexes (46, 47); it appears that the incorporation of heme into apoproteins is strictly required in the pathway for complex assembly (47) and that translated apoproteins for some components are rapidly degraded in the absence of heme for maturation (46). Thus, here in anoxic rice seedlings, the limited availability of heme because of lack of oxygen is most likely responsible for the selective lack of cytochrome pathway components. We showed that low amounts of assembled cytochrome complexes correlate with lower cytochrome absorbance spectra, lower mitochondrial total heme content, and lower total amounts of the heme-containing protein, cytochrome c, in isolated mitochondria. The previous work of Tsuji et al. (23) shows that the transcript abundance for some nuclear encoded subunits of cytochrome oxidase are oxygen-responsive and anoxia-suppressed, whereas the transcripts for mitochondrial encoded subunits are unaffected. This is reminiscent of the situation in heme-deficient yeast mutants (46). We cannot conclude from our current data that all cytochrome-containing complex protein subunits are lower in abundance under anoxia in rice. However, clearly the lower heme content under anoxia correlated with the decreased functional assembly of the cytochrome respiratory pathway complexes in rice and explained the lowered respiratory rate of these mitochondria.

In this set of investigations of the rice proteome it was evident that rice seedlings possess a greatly reduced capacity for both the cytochrome pathway and the alternative pathway under anoxia but did synthesize and assemble most of the remaining components of plant mitochondria in the absence of a functional respiratory chain. The adaptation to oxygen of anoxically grown rice plants involves the synthesis and/or assembly of both the cytochrome and alternative respiratory pathways, whereas growth in air from germination only leads to the cytochrome pathway (Fig. 2B). This raises an interesting perspective on the plant cyanide-resistant alternative oxidase that to our knowledge has not been outlined previously. That is, the alternative oxidase is not only a cyanide-resistant and single-component oxidase, it is also a heme-independent respiratory pathway. Thus, the alternative oxidase could be used following conditions that prevent heme accumulation, such as anoxia, to provide a rapid response to meet the requirements for aerobic respiration and prevent an overreduction of the respiratory chain. There may also be scenarios of heme deficiency in plants that would benefit from a cytochrome-independent respiratory chain, such as metal deficiencies, or a response to heme-synthesis inhibitors, such as certain herbicides. The induction of an alternative oxidase on air adaptation but not during normal air growth from seed also suggests a distinct signaling pathway for the two terminal oxidases. At the transcript level, a calcium dependence of alternative oxidase induction that is not shared by specific nuclear encoded cytochrome
and enhanced ATP production in a timely fashion.

Overall this study showed that the mitochondria synthesized by anoxia-tolerant rice plants (Oryza sativa L. cv. Amaroo), while exhibiting a very low functionality as respiratory organelles, possessed all but a few critical protein complexes for full operation. The mechanisms that maintain the bulk of mitochondrial biogenesis in rice under anoxia are currently elusive but likely contribute to the resilience of this multicellular eukaryote to prolonged anoxia. The subsequent tolerance of rice to post-anoxic shock is likely greatly aided by nearly functional mitochondria that can be engaged to redirect glycolytic flux from ethanol and acetaldehyde to respiratory CO₂ release and enhanced ATP production in a timely fashion.

REFERENCES
1. Wallace, P. G., and Limnane, A. W. (1964) Nature 201, 1191–1194
2. Criddle, R. S., and Schatz, G. (1969) Biochemistry 8, 322–334
3. Hochachka, P. W. (1986) Science 231, 234–241
4. Zhu, H., and Bunn, F. (2001) Science 292, 449–451
5. Chandel, N. S., and Schumacker, P. T. (2000) J. Appl. Physiol. 88, 1880–1889
6. Dagsgaard, C., Taylor, L. E., O’Brien, K. M., and Peyton, R. O. (2001) J. Biol. Chem. 276, 7693–7699
7. Sachs, M. M., Freeing, M., and Okimoto, R. (2000) Cell 20, 761–767
8. Subbaiah, V., and Sachs, M. M. (2003) Ann. Bot. 91, 119–127
9. Gibb, J., Morrell, S., Valdez, A., Setter, T. L., and Greenway, H. (2000) J. Exp. Bot. 51, 785–786
10. Setter, T. L., Ellis, M., Laureles, E. V., Ella, E. S., Senadhira, D., Mishra, S. B., Sarkarung, S., and Datta, S. (1997) Ann. Bot. 79, 67–77
11. Ueda, K., and Tsuji, H. (1971) Protoplasma 73, 203–215
12. Vartapetian, B. B., Maslov, A. I., and Andreeva, I. N. (1975) Plant Sci. Lett. 4, 1–8
13. Costes, C., and Vartapetian, B. B. (1978) Plant Sci. Lett. 11, 115–119
14. Shibasaki, M., and Tsuji, H. (1988a) Plant Physiol. 86, 1098–1102
15. Ushimaru, T., Shibasaki, M., and Tsuji, H. (1992) Plant Cell Physiol. 33, 1065–1071
16. Setter, T. L., and Ella, E. S. (1994) Ann. Bot. 74, 265–271
17. Shibasaki, M., and Tsuji, H. (1988b) Plant Physiol. 86, 1098–1102
18. Bon, Y. C., and Jung, J. (1999) J. Plant Physiol. 155, 255–261
19. Couee, I., Defontaine, S., Carde, J., and Pradet, A. (1992) Plant Physiol. 98, 411–421
20. Vartapetian, B. B., Andreeva, I. N., and Kozhev, G. I. (1976) Protoplasma 88, 215–224
21. Fox, T. C., and Kennedy, R. A. (1991) Planta 184, 510–514
22. Shibasaki, M., and Tsuji, H. (1988b) Plant Cell Physiol. 29, 629–635
23. Tsuji, H., Nakazono, M., Saisho, D., Tsutsumi, N., and Hirai, A. (2000) FEBS Lett. 471, 201–204
24. Tsuji, H., Meguro, N., Suzuki, Y., Tsutsumi, N., Hirai, A., and Nakazono, M. (2003) FEBS Lett. 546, 369–373
25. Heazlewood, J. L., Howell, K. A., Whelan, J., and Millar, A. H. (2003) Plant Physiol. 132, 230–242
26. Hodges, D. M., Delang, J. M., Forney, C. F., and Prange, R. K. (1999) Planta 207, 604–611
27. Appleby, C. A., and Bergersen, F. J. (1980) in Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F. J., ed), pp. 315–335, John Wiley & Sons Ltd, Chichester
28. Chance, B., and Williams, G. R. (1955) J. Biol. Chem. 217, 395–408
29. Humphries, K. M., and Saweda, L. I. (1998) Biochemistry 37, 15835–15841
30. Jansch, L., Kuff, V., Schmitz, U. K., and Braun, H. P. (1996) Plant J. 9, 357–368
31. Sweetlove, L. J., Heazlewood, J. L., Herald, V., Holtzapfel, R., Day, D. A., Leaver, C. J., and Millar, A. H. (2002) Plant J. 32, 1–14
32. Tsuji, H. (1972) Bot. Mag. Tokyo 85, 207–218
33. Huang, S., Greenway, H., and Colmer, T. D. (2003) J. Exp. Bot. 54, 2363–2373
34. Heazlewood, J. L., Tonti-Filippini, J. S., Giege, P., Day, D. A. Whelan, J., and Millar, A. H. (2004) Plant Cell 16, 241–256
35. Millar, A. H., Sweetlove, L. J., Giege, P., and Leaver, C. J. (2001) Plant Physiol. 127, 1711–1727
36. Ebel, H., Jansch, L., and Braun, H. P. (2003) Plant Physiol. 133, 274–286
37. Ebel, H., Heinemeyer, J., and Braun, H. P. (2004) Plant Physiol. 134, 1450–1459
38. de Winde, J. H., and Grivell, L. A. (1993) Prog. Nuc. Acid. Res. Mol. Biol. 46, 51–91
39. Surpin, M., and Chory, J. (1997) Essays Biochem. 32, 113–125
40. Nissli, E., Clementi, E., Paolucci, C., Cuzzi, Y., Tonello, C., Sciorati, C., Bracale, R., Valerio, A., Francoolini, M., Moncada, S., and Carruba, M. O. (2003) Science 299, 896–899
41. Mochihashi, Y., Bewley, J. D., and Yeung, E. C. (1981) Plant Physiol. 68, 318–323
42. Dai, H., Lo, Y. S., Jane, W. N., Lee, W. L., and Chiang, K. S. (1998) Eur. J. Cell Biol. 75, 189–209
43. Logan, D. C., Millar, A. H., Sweetlove, L. J., Hill, S. A., and Leaver, C. J. (2001) Plant Physiol. 125, 662–672
44. Smith, A. G., Marsh, O., and Elder, G. H. (1993) Biochem. J. 292, 503–508
45. Lermontova, I., Kruse, E., Mick, H. P., and Grimm, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8985–8990
46. Saltzgaber-Muller, J., and Schatz, G. (1978) J. Biol. Chem. 253, 305–310
47. Lin, C. I., Golub, E. G., and Beattie, D. S. (1982) Eur. J. Biochem. 128, 309–313
