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Effects of Anoxia on Mitochondrial Biogenesis in Rice Shoots

Modification of *in Organello* Translation Characteristics

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ABSTRACT

Shoots of germinating rice (*Oryza sativa* L.) seedlings are able to grow under anoxia and to withstand long periods of anoxic treatment. Mitochondria were purified from aerobically germinated and anaerobically treated rice shoots by differential and isopycnic centrifugation and were found to consist of two subpopulations. The mitochondrial subpopulation of higher density was used for further characterization. Ultrastructural studies showed anaerobic mitochondria to be significantly different from aerobic mitochondria, with a matrix of lower density and more developed cristae. Aerobic and anaerobic mitochondria also differed in their specific activities for fumarase and succinate dehydrogenase, which were significantly lower after the anoxic treatment. *In vivo* labeling of seedlings with L-[^{35}S]methionine and subsequent isolation of the mitochondria indicated that anoxia induced a drastic decrease, but not a total inactivation, of the synthesis of mitochondrial proteins. *In organello* protein synthesis showed that anaerobic mitochondria were able to synthesize most of the polypeptides synthesized by aerobic mitochondria, although only in the presence of exogenous ATP, as would occur under anoxia. Anaerobic mitochondria, but not aerobic mitochondria, could carry out protein synthesis without a functional respiratory chain. Thus, mitochondrial protein synthesis was found to be potentially functional in the rice shoot under anoxia.

Plant seed germination involves a rapid acceleration of respiratory activity, and thus, all cultivated seeds examined so far have been found to be unable to germinate in the absence of oxygen (5). However, during seedling growth, the developing embryos of different plant species show a number of responses to low partial pressure of oxygen. Thus, the developing embryo of rice (*Oryza sativa* L.) seeds can withstand several days of secondary anoxia, which is applied to aerobically germinated embryos, and shows a significant elongation of the coleoptile under these conditions, whereas the development of the root is stopped (29). Elongation of the coleoptile can even take place under primary anoxia, when the imbibition is carried out under anoxia, thus resulting in anomalous germination because the embryonic root does not grow (29). In contrast, maize root tips are unable to survive for more than 8 to 9 h under anoxia unless they are acclimatized by hypoxic treatment, in which case their survival under anoxia is maintained for at least 22 h (25). Excised pea roots are also unable to survive long periods of anoxia (3).

The ability to sustain active energy metabolism appears to be a major factor of resistance to hypoxia and anoxia. Germinating seeds that can develop a high energy charge under anoxia are less sensitive to the inhibition of germination by hypoxia (2). The high adenylate energy charge obtained in the rice shoot under anoxia is sufficient to allow neosynthesis of proteins and nucleic acids (20, 21, 24). This high adenylate energy charge under anoxia may also contribute to the regulation of cytosolic pH, thus preventing cell death by acidosis (26). However, mere survival after anoxia as determined by the ability to grow after reexposure to air may result from other mechanisms. Thus, the root of 3-d-old rice seedlings can survive long periods of anoxia because it is able to resume growth when the anaerobic treatment is ended (4). Nevertheless, its adenylate energy charge decreases to <0.5 within 1 h of anaerobic treatment and remains at this level for at least 48 h (24).

The ability to retrieve all cellular functions after the anaerobic stress has ended must involve the conservation of at least some DNA-containing organelles in either active or quiescent configuration to allow a rapid return to activity or the biogenesis of new organelles. In growing cultures of facultatively anaerobic yeasts, anoxia appears to impede mitochondrial biogenesis, thus resulting in the dilution of these organelles in the cell population, and induces a massive reorganization of mitochondrial structures resulting in promitochondria (17). These promitochondria seem to be the precursors of functional mitochondria when anaerobically grown yeasts are returned to aerobic conditions (17). In contrast, the measurement of respiration in anaerobic tissues, *in situ* ultrastructural studies, and the isolation of mitochondria from anoxia-treated tissues have shown that mitochondria are conserved in a potentially active configuration in the coleoptile of rice seedlings under anoxia (27, 29, 30).

However, the detection of mitochondrial structures and activities in an anaerobic tissue could be due to either the remaining organelles of a mitochondrial population undergoing continuous degradation or a biogenetically active population of mitochondria. Because the response of the rice shoot

to anoxia allows the continuation of protein and nucleic acid biosynthesis, the hypothesis of mitochondrial biogenesis under anoxia could not be dismissed. In both hypotheses, the mechanisms involved may be specific to a particular set of proteins or genes, thus resulting in anoxia-modified mitochondria showing an array of proteins and, therefore, a range of potential functions different from those of aerobic mitochondria.

MATERIALS AND METHODS

Plant Material and Anaerobic Treatment

Inbred rice (*Oryza sativa* L., var Cigalon) was decontaminated with commercial hypochlorite and rinsed with sterile 0.01 N HCl and sterile water. This procedure did not affect germination but ensured axenic conditions as determined by bacteriological controls. Contaminated batches were discarded. Germination was carried out at 25°C in the dark under axenic conditions in sterile water with vigorous agitation to ensure aerobic conditions. Anaerobiosis was obtained by flushing sterilized and humidified oxygen-free (<20 $\mu L \cdot L^{-1}$) nitrogen at a flow rate of 100 to 200 $mL \cdot min^{-1}$ through an air-tight system connecting the flasks and vials to the nitrogen source. The anaerobic treatment was also performed in the dark. Other details of germination and anoxic treatment were as described previously (20, 21). Rice seedlings aerobically germinated for 48 h were subsequently subjected to anaerobic treatment, which could be therefore described as secondary anoxia as defined by Vartapetian *et al.* (29). Anoxia-treated seedlings were compared with seedlings aerobically germinated for 48 h, because both materials consisted of the same tissues. The comparison of anoxia-treated seedlings with seedlings aerobically grown for the same period would be hampered by the development of primary leaves and secondary roots in the aerobic material.

Preparation of Crude Extracts from Rice Shoots

Shoots were dissected on liquid nitrogen-cooled ice from germinating seedlings that had been subjected to anaerobic treatment and frozen in liquid nitrogen at the end of the anaerobic treatment. These shoots were homogenized at 0 to 4°C in 70 μL per shoot of a medium containing 20 mM Hepes-KOH, 50 mM Mops-KOH (pH 7.8), 20 mM sodium EDTA, 10 mM 2-mercaptoethanol, 100 μM leupeptin, 10% (w/v) glycerol, 0.1% (w/v) fatty acid-free BSA, 0.5% (w/v) PVP, and 0.025% (v/v) Triton X-100. This crude extract was centrifuged at 1700*g* for 5 min. The resulting pellet was reextracted with the above medium. This extraction from the pellet was repeated three times, and the resulting supernatants were pooled with the initial supernatant. Because it interfered with the enzyme assay, 2-mercaptoethanol was omitted from the extraction medium for the measurement of SDH[1] activity.

[1] Abbreviations: SDH, succinate dehydrogenase; AAT, aspartate amino transferase; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; IDH, isocitrate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

Purification of Mitochondria

Mitochondria were prepared by a modification of the procedure described by Douce *et al.* (12) from rice shoots freshly excised from 48-h aerobically germinated seedlings or from seedlings that had been subjected to anaerobic treatment after 48 h of aerobic germination. All homogenization and isolation procedures were performed at 0 to 4°C. Shoots were ground with a mortar and pestle in a medium containing 100 mM Hepes-KOH (pH 7.5), 5 mM sodium EDTA, 300 mM mannitol, 0.05% (w/v) cysteine, 0.1% (w/v) BSA, and 0.6% (w/v) insoluble PVP. The homogenate was filtered through two layers of muslin and two layers of Miracloth (Calbiochem) and then centrifuged at 1700*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 15 min. The resulting pellet was resuspended in a washing medium containing 50 mM Tes-KOH (pH 7.2), 5 mM sodium EDTA, 300 mM mannitol, and 0.1% (w/v) BSA. This suspension was centrifuged at 200*g* for 15 min, and the resulting supernatant was centrifuged at 10,000*g* for 15 min, thus yielding a pellet of crude mitochondria. This pellet was resuspended in 0.5 mL of washing medium and fractionated by isopycnic centrifugation in 26% (v/v) Percoll in washing medium at 40,000*g* for 30 min. Two distinct bands were obtained. Both upper band (F1) and lower band (F2) were collected and diluted 15-fold with washing medium. These suspensions were then centrifuged at 12,000*g* for 15 min. The resulting pellets were concentrated by centrifugation in a minifuge at 10,000*g* for 15 min. A small-scale modification of this method, using minifuge tubes, was used to isolate mitochondria from shoots that had been labeled *in vivo* with L-[^{35}S]methionine as described below. The filtration step was omitted, and a 1-mL 26% (v/v) Percoll gradient was used. The fractions to be collected were determined by comparison with the migration of density marker beads (Pharmacia).

Analysis of Proteins

Protein contents were determined by the method of Bradford (6) using bovine γ-globulin as the standard. The content of BSA in the extraction and washing mediums, expressed as the equivalent in bovine γ-globulin, was subtracted from the total protein content. The added BSA represented 10 to 50% of the total protein measured per sample. The dilution and centrifugation of Percoll-purified mitochondria in the absence of BSA showed that BSA did not bind to the pelleted material.

SDS-PAGE analysis of proteins was carried out in a slab-gel apparatus with gels prepared with 0.1% (w/v) SDS and with acrylamide and *N,N'*-methylenebisacrylamide at concentrations of 15 and 0.08%, respectively. A stacking gel of 5% acrylamide and 0.15% bisacrylamide was used. Protein samples were denatured in the presence of SDS and 2-mercaptoethanol at 100°C for 5 min. Markers of molecular masses 66, 45, 29, 18.4, and 14.3 kD were obtained from Sigma. Polypeptide bands were visualized by fluorography as described by Mocquot *et al.* (21). Other details of SDS-PAGE analysis were as described by McCarthy *et al.* (19).

In Vivo Labeling of Proteins

Batches of 40 to 50 rice seedlings were labeled *in vivo* with 100 μCi of L-[^{35}S]methionine (900–1100 $Ci \cdot mmol^{-1}$) in 5 mL

of sterile water for the final 3 h of germination or anaerobic treatment. The solution of L-[^{35}S]methionine was deaerated by flushing with oxygen-free nitrogen and injected under nitrogen in the vials containing the germinating seedlings. The labeling was ended by washing the seedlings with deaerated 100 mM methionine. The shoots were excised in a 7-L glovebox, under a 200 mL·min^{-1} flow of nitrogen, and immediately homogenized for the isolation of mitochondrial fractions as described above. The isolated mitochondrial fractions were frozen in liquid nitrogen and kept at −80°C until the measurement of radioactivity.

In Organello Mitochondrial Protein Synthesis

A modification of the method described by Forde *et al.* (13) was used. Purified mitochondria obtained in fraction F2 (approximately 15 μg protein) were incubated in the presence of 20 μCi L-[^{35}S]methionine (950–1200 Ci·mmol^{-1}) in a final volume of 250 μL. The basic incubation medium contained 240 mM mannitol, 72 mM KCl, 0.8 mM EGTA, 20 μM of each amino acid (minus methionine), 8 mM Tricine-KOH, and 4 mM KH_2PO_4-KOH (pH 7.2). In addition, one of the following mixtures was included: (a) 8 mM succinate or NADH or acetate, 1.6 mM GTP, 1.6 mM ADP, 24 mM $MgCl_2$, 8 mM glucose, and 24 units·mL^{-1} hexokinase or (b) 4.8 mM ATP, 0.4 mM GTP, 6 mM $MgCl_2$, 6.4 mM phosphocreatine, and 16 units·mL^{-1} creatine phosphokinase. The incubation was carried out under atmospheric air at 25°C. The time course of the reaction was followed by removing 40-μL samples which were treated with 60 mM methionine. The radioactive label incorporated in the TCA-precipitable material was determined as described below. Incorporation at zero time was determined by adding the radiolabeled methionine to denatured mitochondria in the reaction medium supplemented with 60 mM nonradioactive methionine. Background (blank filters) was typically 100 to 200 cpm, and zero times were 500 to 800 cpm/10 μL of reaction mixture. Incorporation values for 60 min at 25°C were 4000 to 35,000 cpm. Incorporation was linear for up to 120 min. Steady-state velocities of incorporation were measured. Preparations of mitochondrial translation products were obtained by *in organello* protein synthesis for 180 min in the presence of exogenous ATP. An aliquot was used for the determination of incorporated radiolabel, and the preparation was frozen in liquid nitrogen and kept at −80°C until analysis by SDS-PAGE.

Measurement of Radioactivity

Aliquots of the mitochondrial fractions (1–5 μL) were directly measured in ACS (Amersham) scintillation liquid for the determination of the total radioactivity. Other aliquots (5–10 μL in the case of *in vivo* labeling or 36 μL in the case of *in organello* labeling) were precipitated with 10% (w/v) TCA and 1 mM methionine in the presence of 0.05 mg·mL^{-1} carrier BSA and heated at 100°C for 15 min. The cooled precipitate was collected on a glass fiber filter (Whatman GF/C) and washed with 5% (w/v) TCA, 1 mM methionine, and then 70% ethanol. The filter was humidified with 150 μL of water and solubilized in the presence of 1 mL of Protosol (Dupont). The radioactivity was then measured in ACS (Amersham) scintillation liquid for the determination of incorporation.

EM

The mitochondria were incubated for 2 h at 4°C in BSA-free washing buffer containing 2.5% glutaraldehyde (agar aids). After centrifugation at 10,000*g* for 10 min, the mitochondrial pellets were postfixed with 1% osmium tetroxide in 100 mM phosphate buffer, pH 7.2, containing 0.3 M mannitol for 4 h at 4°C, and then rinsed with the same buffer without mannitol. The mitochondria were then treated with 1% tannic acid (Mallinckrodt) (7) for 30 min at 20°C, rinsed with distilled water, covered with 3% agar, and dehydrated with ethanol and propylene oxide. After the mitochondria were embedded in Epon, the ultrathin sections were collected on plain 600-mesh copper grids, successively contrasted with ethanolic uranyl acetate and aqueous lead citrate, and then observed with a Philips CM10 electron microscope.

Enzyme Activities

All enzyme activities were assayed spectrophotometrically at 30°C according to previously published methods. All assays were first performed on blanks. Activities were linear with respect to time for at least 1 min and were proportional to the amounts of sample protein added to the assay. The activities of NAD-dependent GDH (EC 1.4.1.2.), NAD-dependent IDH (EC 1.1.1.41), and G6PDH-6PGDH (EC 1.1.1.49 and EC 1.1.1.44, respectively) were assayed by following the reduction or the oxidation of the cosubstrate as described by McCarthy *et al.* (19), Cox and Davies (10), and Douce *et al.* (12), respectively. The assay of G6PDH-6PGDH activity in the absence or in the presence of Triton X-100 allowed the determination of the immediate activity and of the latent, membrane-enclosed activity as described by Douce *et al.* (12). Fumarase (EC 4.2.1.2), SDH (EC 1.3.5.1), and AAT (EC 2.6.1.1.) activities were assayed as described by Hill and Bradshaw (14), Maeshima *et al.* (18), and Rej and Horder (23), respectively.

RESULTS

Effects of Anoxia on the Development of Rice Seedlings

Rice seedlings that had been germinating for 48 h under aerobic conditions were transferred to anaerobic conditions. The growth of the primary root was stopped, whereas the shoot was found to continue to grow, as shown in Table I. The survival of the seedlings under anoxia was examined by transferring the anoxia-treated seedlings back to aerobic conditions. As shown in Table I, the return to air resulted in root and shoot growth with the emergence of secondary roots and primary leaves after an anaerobic treatment of up to 19 d. Furthermore, the anaerobic elongation of the shoot occurred in parallel with the increase of protein in this organ (Fig. 1), as determined by the measurement of protein in crude extracts from anoxia-treated shoots.

Table I. *Effects of Secondary Anoxia on the Germination and Growth of Rice Seedlings*

Time under Secondary Anoxia (d)	Growth under Secondary Anoxia of 48-h Aerobically Germinated Seedlings			Growth after 2 d of Return to Air		
	Root length	No. of secondary roots	Shoot length	Root length	No. of secondary roots	Appearance of primary leaves
	mm		*mm*	*mm*		
0	3.4 ± 0.6 (n = 28)	0 (n > 100)	4.0 ± 0.7 (n = 40)			
2	4.1 ± 0.8 (n = 50)	0 (n > 100)	11 ± 1 (n = 50)	12.7 ± 0.6 (n = 7)	2–5 (n = 7)	Yes (n = 17)
11	3 ± 1 (n = 6)	0 (n > 100)	23 ± 5 (n = 50)	8.7 ± 1.5 (n = 6)	2–3 (n = 6)	Yes (n = 6)
19	3 ± 1 (n = 11)	0 (n = 11)	26 ± 5 (n = 11)	6 ± 1[a] (n = 11)	2–4[a] (n = 11)	Yes (n = 11)

[a] Mean ± SE. Growth after 3 d.

Detection of Mitochondrial Enzyme Activities

To determine the presence of enzyme activities in anoxia-treated rice shoots, the vials containing the rice seedlings were frozen in liquid nitrogen immediately after elimination of the germination water and were kept frozen for 1 to 5 d at −40°C. The enzyme activities were measured with total extracts from shoots that had been excised on liquid nitrogen-cooled ice. In Figure 2 is shown the time course of the effects of anoxia on the specific activities of NAD-dependent GDH, NAD-dependent IDH, SDH, and fumarase which are associated with the mitochondrial compartment and of aspartate aminotransferase which is present in both the cytosol and the mitochondrial matrix (11).

The four mitochondria-specific activities under study could be detected throughout the 15 d of anaerobic treatment. However, anoxia did not have the same effects on these activities. The specific activities of the NAD-dependent dehydrogenases decreased sharply during the first 5 h of the anaerobic treatment and then leveled off at about 15% of the initial activity. In contrast, the specific activities of fumarase and SDH showed a smaller decrease and leveled off at about 50% of the initial activities. These changes in mitochondrial enzyme activities contrasted with the stability of the cytosolic and mitochondrial AAT specific activity throughout the anaerobic treatment.

Because the effects of anoxia on mitochondrial enzyme activities appeared to have leveled off after 24 h of treatment, it was expected that anoxia-modified mitochondria could be obtained from rice seedlings that had been subjected to 48 h of anaerobic treatment.

Characterization of Anoxia-Modified Mitochondria

Mitochondria were purified from rice shoots by differential and isopycnic centrifugation. The excision of anoxia-treated shoots was carried out under atmospheric air. However, during the excision, anoxia-treated seedlings and excised shoots were kept under conditions of low oxygen pressure to minimize the effects of the return to air until the tissue was disrupted and diluted in a large volume of extraction buffer. Subsequent steps were carried out under atmospheric air. The purification was followed by measuring fumarase activity as a marker of the mitochondrial matrix, SDH activity as a marker of the mitochondrial inner membrane, immediate G6PDH-6PGDH activity as a marker of the cytosol and broken plastids, and latent G6PDH-6PGDH activity as a marker of the plastidial matrix (12).

Percoll gradient isopycnic centrifugation showed that mitochondria from rice shoots were heterogeneous. Both aerobic and anoxia-treated shoots yielded two subpopulations of mitochondria of respective apparent density in the presence of 0.25 M sucrose of 1.042 to 1.049 (fraction F1) and 1.057 to 1.070 (fraction F2). The purification results are given in Table

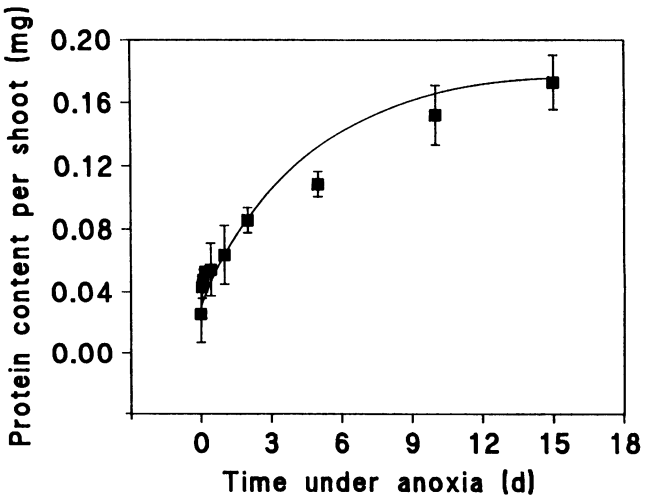

Figure 1. Effects of secondary anoxia on the protein content of rice shoots. Shoots were dissected from 48-h aerobically germinated seedlings that had been subjected to various periods of secondary anoxia. Crude extracts were prepared as described in "Materials and Methods." The protein content of crude extracts of the shoots was determined. The results are the means (± SE) of three independent experiments.

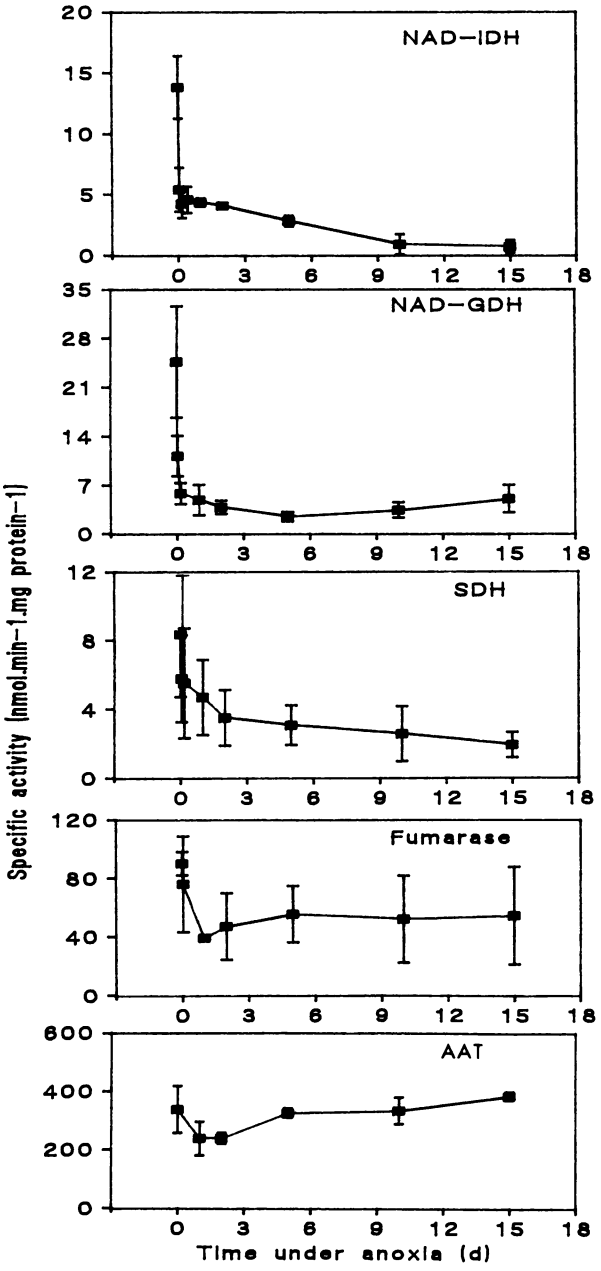

Figure 2. Effects of secondary anoxia on the activities of mitochondrial enzymes. Shoots were dissected from 48-h aerobically germinated seedlings that had been subjected to various periods of secondary anoxia. The enzyme activities in the crude extracts were determined. The results are the means (± SE) of three independent experiments.

II. Fraction F2 was less contaminated by cytosolic and plastidial activities than fraction F1 and had the higher specific activities for the mitochondrial enzyme markers.

Electron micrographs of aerobic and anaerobic fraction F2 preparations are shown in Figure 3. A large majority of organelles were recognized as mitochondria. Intact etioplasts were detected in both aerobic and anaerobic preparations but represented <1% of the total organelles in the aerobic preparation and approximately 5% in the anaerobic preparation. Intact bacteria were not detected. Aggregated material with no organellar structure could be detected in both preparations. A number of ultrastructural differences were observed between aerobic and anaerobic mitochondria. The matrix of anaerobic mitochondria showed a lower density than that of aerobic mitochondria. In contrast, the inner membrane of anaerobic mitochondria had more cristae and complex invaginations than that of aerobic mitochondria.

In addition to these ultrastructural differences, these aerobic and anaerobic mitochondria of higher density also differed in their specific activities for fumarase and SDH (Table II). Thus, the specific activity of SDH was decreased fourfold under the effects of anoxia.

The mitochondrial protein content per shoot was estimated from the total activities of fumarase and SDH in the total extract and the specific activities of these two enzymes in the purified mitochondria obtained in fraction F2. As shown in Table III, the estimations obtained with the two enzyme markers showed a consistent trend, suggesting that the mitochondrial protein content increased during the first 48 h of the anaerobic treatment.

Incorporation of L-[^{35}S]Methionine into Mitochondrial Proteins

Rice seedlings were incubated with L-[^{35}S]methionine for the last 3 h of aerobic germination or of anaerobic treatment. The labeled shoots were excised and disrupted in a homogenization buffer after rinsing the seedlings with 100 mM nonradioactive methionine. These steps were carried out in a glovebox under a flow of nitrogen. Similar results were obtained when the radioactive labeling was terminated by adding deaerated nonradioactive methionine directly in the N_2-flushed vial. The small-scale method described in "Materials and Methods" was used to isolate the F1 and F2 fractions.

The effects of anoxia on the total radioactivity and the incorporation of the radiolabeled amino acid in the F1 and F2 fractions are shown in Figure 4. The values of total radioactivity and incorporation can be compared with the values reported by Ricard and Pradet (24) for Tris-soluble extracts of rice seedlings. Given that the protein content per shoot is about 30 μg after 48 h of aerobic germination and 70 μg after 24 h of subsequent anaerobic treatment, the values of uptake into the shoot measured by these authors corresponded to 31,000 cpm · μg protein^{-1} under aerobic conditions and 3400 cpm · μg protein^{-1} after 24 h of anaerobic conditions. The values of total radioactivity presented here are 60 times lower in the case of aerobically grown seedlings and 40 times lower in the case of anaerobically treated seedlings. This large difference probably results from the washing of metabolites from the mitochondria during the purification.

Table II. *Purification of Mitochondria from Rice Shoots*

Mitochondria were purified (A) from rice shoots dissected from 48-h aerobically germinated seedlings or (B) from 48-h aerobically germinated rice seedlings which were subsequently subjected to 48 h of anoxic treatment. The results are the means of three to four independent experiments. ND, Not determined.

	Total Protein	Specific Activity				Yield Fumarase
		Immediate G6PDH-6PGDH	Latent G6PDH-6PGDH	SDH	Fumarase	
	mg		*nmol·min⁻¹·mg⁻¹*			%
A						
Total extract	60	ND	ND	8	85	100
Crude extract	40	73	3	ND	44	35
Crude mitochondria	1	6	27	8.5	109	2
Fraction F1	0.3	27	5.3	1	106	0.6
Fraction F2	0.1	1.6 ≪ 2.6	<1	26	250	0.5
B						
Total extract	105	ND	ND	4	50	100
Crude extract	70	118.5	1.5	ND	38	51
Crude mitochondria	0.5	26	24	ND	93	1
Fraction F1	0.3	13	6	1	75	0.4
Fraction F2	0.1	0	8	7	166	0.3

As shown in Figure 4, L-[^{35}S]methionine was found to be incorporated into the proteins of fractions F1 and F2 before and during the anaerobic treatment. The observed incorporation under aerobic conditions was not correlated with the level of nonmitochondrial enzyme markers in fractions F1 and F2 (Table II). Furthermore, EM of aerobic F2 fractions showed the absence of bacterial contamination. The observed incorporation could, therefore, be ascribed, at least partially, to mitochondrial proteins.

The anoxic treatment was found to induce a rapid and drastic decrease of incorporation, after which the incorporation remained constant at a level fivefold lower than that found under aerobic conditions. The anaerobic incorporation into the two fractions was approximately the same. The decrease of incorporation under anoxia was similar to the effects of anoxia on the synthesis of Tris-soluble proteins reported by Ricard and Pradet (24).

However, the residual incorporation observed under anoxia could have resulted from nontranslational binding of the radioactive marker to proteins. The total amount of incorporated radioactivity into the proteins of F1 and F2 under anoxia represented only 1% of the uptake by anaerobic shoots, taking into account the yield of mitochondrial markers in fractions F1 and F2. The incubation of denatured mitochondrial proteins (6 mg·mL^{-1}) and L-[^{35}S]methionine (0.02 μCi·mL^{-1}) resulted in the incorporation of 1 cpm·μg protein^{-1}, which was 100 times lower than the observed incorporation under anoxia. EM of anaerobic F2 fractions showed the absence of bacterial contamination, and the observed incorporation was not correlated with the level of free G6PDH-6PGDH contamination (Table II). Furthermore, because the level of plastid contamination in fraction F2 was lower than 5% as shown by EM, the residual incorporation under anoxia could be ascribed, at least partially, to mitochondrial proteins.

The uptake difference between aerobically grown and anoxia-treated shoots (24) implies that the amount of L-[^{35}S] methionine available for protein biosynthesis in the cytosolic compartment is lower under conditions of anoxia. Thus, the real incorporation under anoxia is probably underestimated relative to that under aerobic conditions. This underestimation might also be compounded by the degradation of proteins, if the inactivation described in "Detection of Mitochondrial Enzyme Activities" was due to proteolysis.

Mitochondrial Protein Synthesis

In organello protein synthesis was carried out with the mitochondrial population of higher density (fraction F2). Controls using acetate as energy substrate and erythromycin as inhibitor of bacterial protein synthesis showed that the observed protein synthesis was not due to bacteria (Table IV). Controls using chloramphenicol as inhibitor of prokaryotic protein synthesis and cycloheximide as inhibitor of eukaryotic protein synthesis showed that the observed protein synthesis was not due to eukaryotic translation (Table IV). Because the level of plastid contamination in fraction F2 was lower than 5% as shown by EM, the observed prokaryotic protein synthesis could be ascribed mainly to mitochondria. Erythromycin has been reported to affect chloroplastic protein synthesis, but not mitochondrial protein synthesis, in tissue cultures of *Nicotiana sylvestris* (28). The absence of inhibition by 75 μg·mL^{-1} erythromycin (Table IV) also indicated that the observed protein synthesis was due to mitochondria.

Aerobic mitochondria were found to synthesize polypeptides actively with exogenous NADH or succinate as respiratory substrates in the presence of ADP (Table V). This protein synthesis was completely inhibited by KCN (Table V). Cyanide concentrations of 250 μM and 1 mM gave, respectively, 50 and 92% inhibition of mitochondrial protein synthesis (data not shown). These concentrations were of the same order of magnitude as those necessary for the inhibition of mitochondrial protein synthesis in castor bean mitochondria

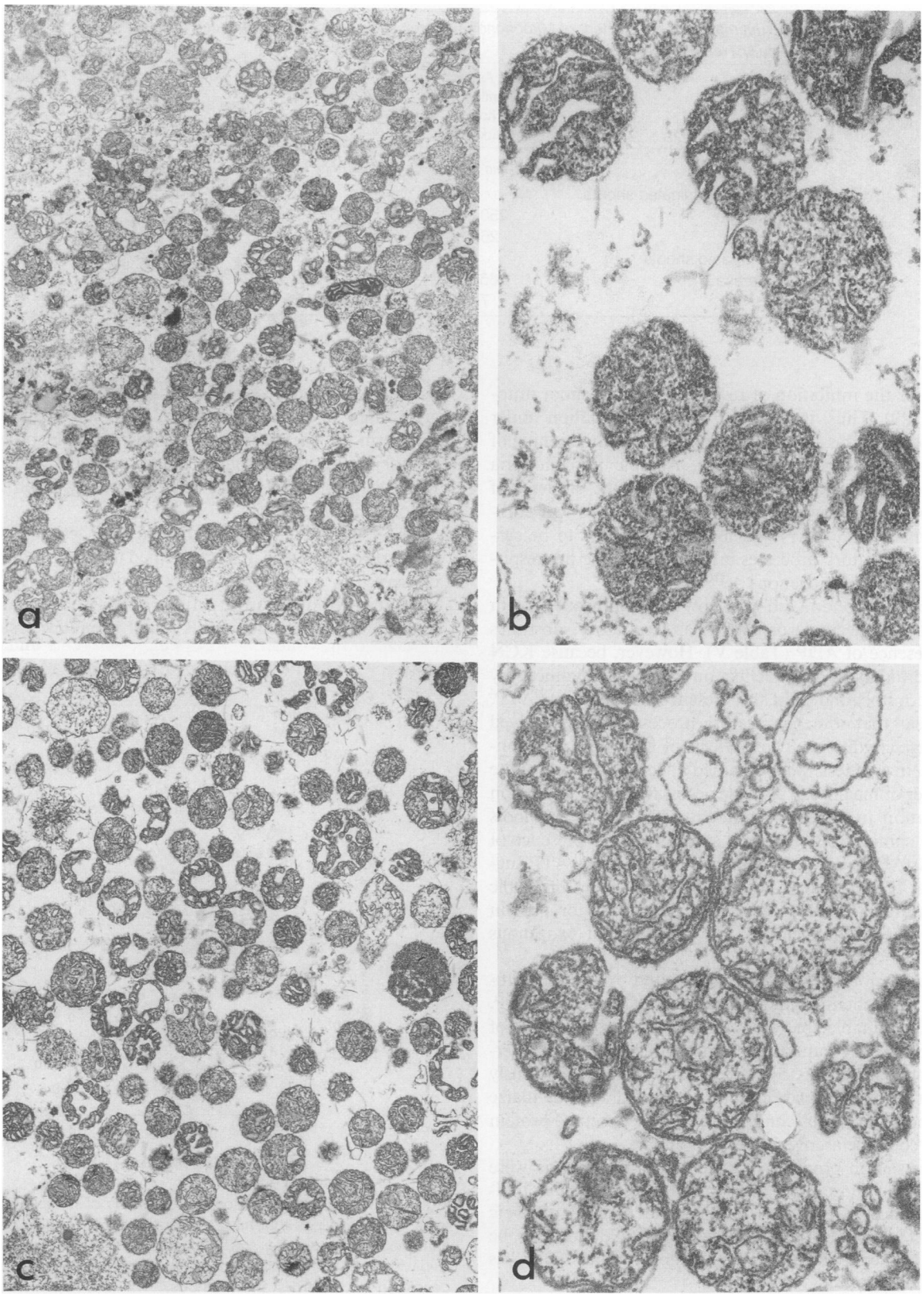

Figure 3. Electron micrographs of mitochondria purified from rice shoots. The mitochondria isolated in fraction F2 were analyzed by EM. The preparations isolated from 48-h aerobically germinated rice seedlings and from 48-h aerobically germinated rice seedlings that had been subjected to 48 h of anaerobic treatment are represented in a–b and c–d, respectively (a and c, ×10,600; b and d, ×41,800). Nonorganellar contaminants were present as well as a few plastids. The matrix of anaerobic mitochondria (d) is less dense than that of aerobic mitochondria (b), but the cristae are more developed.

Table III. *Estimation of the Content of Mitochondrial Proteins in Aerobically Germinated and Anaerobically Treated Rice Shoots*

	Specific Activity in Purified Mitochondria (fraction F2)	Total Activity per Coleoptile	Total/Specific Activity
	nmol·min⁻¹·mg⁻¹	*nmol·min⁻¹*	*μg protein*
Aerobically germinated shoots			
Fumarase	250	3.6	14.4
SDH	26	0.3	11.5
Anoxia-treated shoots			
Fumarase	166	4	24
SDH	7	0.3	46

(15) and for the inhibition of respiration in rice shoot mitochondria (29). Thus, it appeared that the translation under these conditions was strictly dependent on the functioning of the CN-sensitive respiratory chain. Aerobic mitochondria were also able to synthesize polypeptides in the presence of exogenous ATP. However, because this synthesis was completely inhibited by KCN (Table V), it appeared to be sustained by respiratory processes rather than by the nonrespiratory utilization of exogenous ATP.

Anoxic mitochondria did not show any significant protein synthesis with succinate or NADH as respiratory substrates in the presence of ADP (Table V). However, because KCN was found to have a drastic effect on the protein synthesis by anaerobic mitochondria in the presence of exogenous ATP, it was clear that some respiratory processes were involved under these conditions. These effects of KCN on mitochondrial protein synthesis were obtained at concentrations of the same order of magnitude as those necessary for the inhibition of respiration in anaerobic mitochondria from rice shoots (29). The comparison of protein synthesis in the presence of exogenous ADP and in the presence of exogenous ATP suggested that the respiratory processes carried out by anaerobic mitochondria did not produce sufficient ATP for protein synthesis but might facilitate the utilization of exogenous ATP.

The translation products of aerobic and anaerobic mitochondria were obtained under conditions of exogenous ATP, which were suitable for protein synthesis by both types of mitochondria. The translation products of aerobic and anaerobic mitochondria (Fig. 5) were similar to those previously found for better described monocot material such as maize mitochondria (16), thus confirming that the observed protein synthesis could be ascribed to mitochondria.

The translation products of anaerobic mitochondria were comparable to those from aerobic mitochondria, thus indicating that most mRNA were retained after 48 h of anaerobic treatment. Some differences of expression could, however, be detected between aerobic and anaerobic mitochondria, as indicated in Figure 5.

DISCUSSION

EM studies *in situ* of rice shoots from seedlings that had been transferred to anoxia after 6 d of germination under aerobic conditions have shown that mitochondrial structures are conserved for at least 5 d of anaerobic treatment (29). Furthermore, the anaerobically grown rice coleoptile has respiratory activity immediately after return to air and shows a weak signal of cytochrome oxidase activity when analyzed histochemically (30).

The detection of mitochondrial enzyme activities described in the present work (Fig. 2) confirmed that mitochondria were conserved in a potentially active form in rice shoots of germinating seedlings during periods of anaerobic treatment up to 15 d. However, anoxia was found to induce a significant

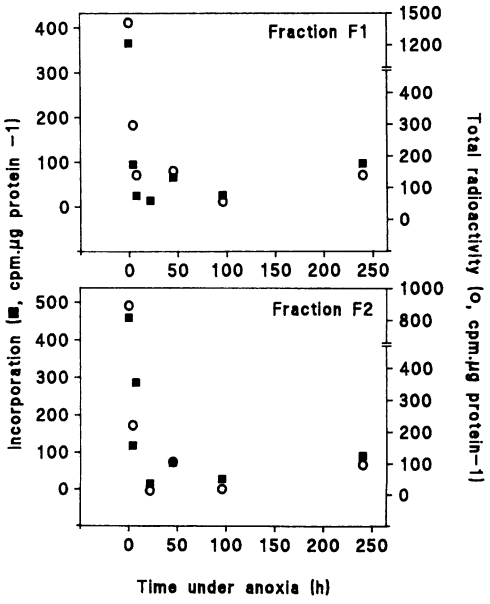

Figure 4. Effects of anoxia on the synthesis of mitochondrial proteins. Rice seedlings that had been subjected to various periods of anaerobic treatment after a 48-h aerobic germination were labeled *in vivo* with L-[^{35}S]methionine. Rice shoot mitochondria were purified as described in "Materials and Methods." The amounts of radioactive label present in the mitochondrial preparation (total radioactivity) and incorporated in TCA-precipitated material (incorporation) were determined in the two fractions, F1 and F2, which were collected from the Percoll gradient. The results show one typical experiment from three concordant experiments.

Table IV. *Effects of Inhibitors on Mitochondrial Protein Synthesis by Rice Shoot Mitochondria*

In organello protein synthesis was carried out with crude or fraction F2 aerobic mitochondria and with fraction F2 anaerobic mitochondria. Aerobic mitochondria were incubated in the presence of succinate as respiratory substrate or in the presence of an exogenous ATP-generating system, whereas anaerobic mitochondria were incubated only in the presence of an exogenous ATP-generating system. The results are the means of two to seven independent experiments. When there are only two results, both are given. The results of a preliminary experiment with crude mitochondria using succinate as a source of energy are also given. ND, Not determined.

	Steady-State Velocity of Incorporation		
	Aerobic mitochondria		Anaerobic mitochondria (Exogenous ATP)
	Succinate	Exogenous ATP	
	Crude mitochondria		Fraction F2
	$cpm \cdot h^{-1} \cdot \mu g\ protein^{-1}$		
Control	6,500	500 ± 100	3,000 ± 900
+Chloramphenicol (100 $\mu g \cdot mL^{-1}$)	200	0–0	0–0
+Erythromycin (6 $\mu g \cdot mL^{-1}$)	ND	500 ± 100	2,400–3,400
+Erythromycin (75 $\mu g \cdot mL^{-1}$)	6,684	ND	ND
	Fraction 2		Fraction F2
Control	15,000 ± 1,000	3,900 ± 500	3,000 ± 900
+Cycloheximide (10 $\mu g \cdot mL^{-1}$)	ND	4,100 ± 400	2,800 – 3,400
Acetate replacing succinate	170–180		

inactivation of these mitochondrial enzyme activities. This inactivation was faster than the rate of protein synthesis, thus indicating that it did not result exclusively from the dilution of mitochondrial proteins in the elongating cell. The decrease of enzyme activities showed some specificity because AAT specific activity was found to remain constant throughout the anaerobic treatment. Furthermore, the NAD-dependent dehydrogenases showed a more drastic decrease than fumarase and SDH.

The present work showed that Percoll-purified mitochondria could be isolated from anaerobically treated rice shoots (Table II, Fig. 3). The monitoring of the purification of mitochondria showed that crude mitochondria preparations such as those used in previous studies (27, 30) were enriched in plastidial activities and also contained nonorganellar contaminating material. Isopycnic centrifugation showed a heterogeneity of density among aerobic and anaerobic mitochondria. This heterogeneity should be investigated further to determine whether it results from artifactual, cellular, or subcellular heterogeneity.

In excised pea root, the anaerobic degradation of mitochondria results in the clarification of the matrix and the reduction of cristae (3). In the present work, EM showed that anaerobic mitochondrial vesicles from rice shoots had a more developed inner membrane, which is consistent with *in situ* observations (29). In contrast, the specific activity of the inner membrane enzyme SDH was found to decrease under the effects of anoxia. Furthermore, the respiratory activity of crude mitochondria with succinate or NADH as substrate has been reported to be lower after an anaerobic treatment (30). Thus, the apparent increase of inner membrane surface in the mitochondria isolated from anoxia-treated rice shoots might be correlated to nonrespiratory functions rather than to respiratory activity or to degradative processes.

The decrease in SDH activity under the effects of anoxia observed in the present work (Fig. 2) is consistent with the decrease in succinate respiration reported by Vartapetian *et al.* (30). Furthermore, aerobic and anaerobic mitochondria showed profound differences in the utilization of energy sources for protein synthesis (Tables IV and V). Thus, in contrast with aerobic mitochondria, anaerobic mitochondria could not sustain protein synthesis with oxidative phosphorylation. Because isolation procedures involved some degree of return to atmospheric air, the mitochondrial fraction isolated from anoxia-treated shoots could have lost some of the properties that anoxia might induce. It appeared, however, that the anaerobic mitochondria obtained in the present work had been structurally and functionally modified by the anoxic treatment.

The detection of mitochondrial enzyme activities undergoing inactivation did not imply that mitochondrial biogenesis was maintained under anoxia, because these activities could be due to the remaining organelles of a mitochondrial population undergoing continuous degradation or mere dilution in the elongating cell under the effects of anoxia. Thus, Vartapetian *et al.* (29, 30) reported that there was no neoformation of respiratory enzymes in rice coleoptiles under anoxia.

The neosynthesis of soluble proteins in rice shoots under

Table V. *Utilization of Energy Sources by Aerobic and Anaerobic Mitochondria for in Organello Protein Synthesis*

In organello protein synthesis was carried out with fraction F2 aerobic and anaerobic mitochondria in the presence of respiratory substrates such as succinate or exogenous NADH or in the presence of an exogenous ATP-generating system. The contribution of the CN-sensitive respiratory chain was determined from the effects of 2 mM KCN. The results are the means of two to seven independent experiments. ND, Not determined.

	Steady-State Velocity of Incorporation	
	Aerobic mitochondria	Anaerobic mitochondria
	$cpm \cdot h^{-1} \cdot \mu g\ protein^{-1}$	
Succinate	15,000 ± 1,000	0–300
Succinate + KCN	150 ± 50	0–0
NADH	3,800 ± 500	0–0
NADH + KCN	0–0	
Exogenous ATP	3,900 ± 500	3,000 ± 900
Exogenous ATP + KCN	80 ± 20	1,000 ± 150

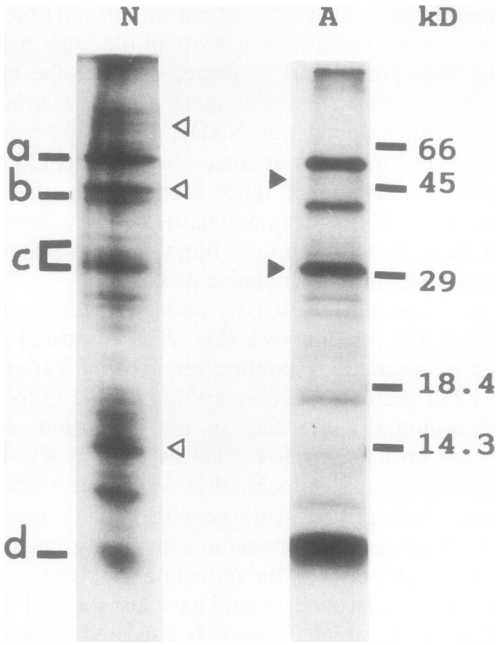

Figure 5. Effects of anoxia on mitochondrial protein synthesis. Polypeptides synthesized by purified rice shoot mitochondria (fraction F2) in the presence of exogenous ATP were separated by SDS-PAGE (15% homogeneous gel) and visualized by fluorography. Identical amounts of incorporated radioactivity (20,000 cpm) were loaded on each track. Lanes: N, mitochondria isolated from 48-h aerobically germinated seedlings; A, mitochondria isolated from 48-h aerobically germinated seedlings that were subsequently subjected to 48 h of anoxic treatment. The putative identity of the mitochondrially synthesized polypeptides (indicated on left) was assigned from the relative migrations of the homologous maize mitochondrial gene products in 15% homogeneous SDS-PAGE (16): a, F1 α-ATPase; b, ribosomal protein; c, cytochrome *c* oxidase subunits (?); d, dicyclohexylcarbodiimide-binding protein. Open and solid arrowheads, positions of some aerobiosis-specific and anoxia-induced, or anoxia-enhanced, polypeptides, respectively.

anoxia has been demonstrated (21, 24), thus indicating that the cytosolic translational machinery is active in rice shoots under anoxia and may produce nuclear-encoded mitochondrial proteins. In the present work, the content of mitochondrial protein per shoot was found to increase after 48 h of anaerobic treatment (Table III). *In vivo* labeling also showed that, under anoxia, L-[^{35}S]methionine was incorporated into the TCA-precipitated material of mitochondrial preparations (Fig. 4). Although these results must be treated with caution, for the reasons discussed in "Results," they suggest that anoxia did not abolish the neosynthesis of mitochondrial proteins.

In organello protein synthesis showed that anaerobic mitochondria retained the capacity to translate organellar mRNA (Fig. 5). The pattern of translation products was very similar to that obtained with aerobic mitochondria, which shows that most mRNA were retained under anoxia. However, major polypeptides such as those of molecular masses 70, 42, and 14 kD were not synthesized anymore by anaerobic mitochondria. In contrast, one minor additional polypeptide of 50 kD was detected in the translation products of anoxic mitochondria, and some polypeptides, such as the polypeptide of 40 kD, appeared to be more actively synthesized by anaerobic mitochondria. These results indicate that the expression of mitochondrially encoded polypeptides was modified by anoxia. The expression of mitochondrially encoded polypeptides has been found to be affected by environmental and developmental factors in a number of cases such as the effects of aging on Jerusalem artichoke mitochondria (13), the effects of heat shock on corn mitochondria (22), the effects of nuclear background on teosinte mitochondria (9), and the effects of chronic ethanol consumption on rat liver mitochondria (8). The present work gives a further example of changes in the expression of mitochondrially encoded polypeptides induced by an environmental stress.

These translation products were obtained under conditions in which the contribution of respiratory processes was important (Table V). However, because respiration itself could not sustain protein synthesis in anaerobic mitochondria, these respiratory processes, which were mainly CN sensitive, could be thought to facilitate the import of exogenous ATP, which could be carried out through the energy-linked mechanism of net uptake of adenine nucleotides described by Abou-Khalil and Hanson (1) in corn mitochondria. It appeared that anaerobic mitochondria could carry out protein synthesis with an influx of exogenous ATP. Mitochondrial protein synthesis could, therefore, take place under anoxia if the influx of ATP was sufficient. Anaerobic mitochondria showed a significant level of protein synthesis in the presence of 2 mM KCN. This could not be due to the functioning of the alternative respiration pathway, because the mitochondria isolated from anaerobically grown rice shoots are inhibited >90% by KCN (29). This protein synthesis in the presence of 2 mM KCN could, therefore, be assigned to a nonrespiratory influx of ATP, as would occur under anoxia. In contrast, aerobic mitochondria could not sustain protein synthesis without a functional CN-sensitive respiratory chain. However, the observed protein synthesis by anaerobic mitochondria in the presence of 2 mM KCN might be overestimated relative to *in vivo* conditions because the exogenous concentration of ATP in the present experiments was high.

Anaerobic mitochondria were, therefore, potentially able to carry out protein synthesis with a nonrespiratory influx of ATP, in contrast with aerobic mitochondria, and to express most of the mitochondrial polypeptides found in aerobic mitochondria. Thus, the polypeptides putatively ascribed to the α-subunit and the dicyclohexylcarbodiimide-binding subunit of ATPase, to a ribosomal protein, and to subunits of cytochrome *c* oxidase could be expressed by anaerobic mitochondria. Anoxia, however, inhibited the expression of some major polypeptides and induced or facilitated the expression of other polypeptides such as the putative ribosomal protein. Thus, mitochondrial protein synthesis appeared to be potentially functional under anoxia, which is consistent with the existence of biogenetically active mitochondria. However, the modification of protein expression and the differential inactivation of mitochondrial enzymes may only allow the rapid reactivation of mitochondrial functions after the anaerobic stress has ended. Thus, further work is required to identify anoxia-induced mitochondrial proteins and to determine

whether mitochondria have a physiological function under anoxia.

LITERATURE CITED

1. **Abou-Khalil S, Hanson JB** (1979) Energy-linked adenosine diphosphate accumulation by corn mitochondria. I. General characteristics and effects of inhibitors. Plant Physiol **64:** 276–280
2. **Al Ani A, Bruzau F, Raymond P, Saint-Ges V, Leblanc J-M, Pradet A** (1985) Germination, respiration, and adenylate energy charge of seeds at various oxygen partial pressures. Plant Physiol **79:** 885–890
3. **Andreev VY, Generozova IP, Vartapetian BB** (1991) Energy status and mitochondrial ultrastructure of excised pea root at anoxia and postanoxia. Plant Physiol Biochem **29:** 171–176
4. **Bertani A, Brambilla I, Menegus F** (1980) Effects of anaerobiosis on rice seedlings: metabolic rate and fate of fermentation products. J Exp Bot **120:** 325–331
5. **Bewley JD, Black M** (1985) Seeds: Physiology of Development and Germination. Plenum Press, New York
6. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
7. **Carde JP** (1987) Electron microscopy of plant cell membranes. Methods Enzymol **148:** 599–622
8. **Coleman WB, Cunningham CC** (1990) Effects of chronic ethanol consumption on the synthesis of polypeptides encoded by the hepatic mitochondrial genome. Biochim Biophys Acta **1019:** 142–150
9. **Cooper P, Newton KJ** (1989) Maize nuclear background regulates the synthesis of a 22-kDa polypeptide in *Zea luxurians* mitochondria. Proc Natl Acad Sci USA **86:** 7423–7426
10. **Cox GF, Davies DD** (1967) Nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase from pea mitochondria: purification and properties. Biochem J **105:** 729–734
11. **Douce R** (1985) Mitochondria of Higher Plants: Structure, Function and Biogenesis. Academic Press, New York
12. **Douce R, Bourguignon J, Brouquisse R, Neuburger M** (1987) Isolation of plant mitochondria: general principles and criteria of integrity. Methods Enzymol **148:** 403–415
13. **Forde BG, Oliver RJ, Leaver CJ** (1979) *In vitro* study of mitochondrial protein synthesis during mitochondrial biogenesis in excised plant storage tissue. Plant Physiol **63:** 67–73
14. **Hill RL, Bradshaw RA** (1969) Fumarase. Methods Enzymol **13:** 91–99
15. **Kaderbhai N, Beechey RB, Kaderbhai MA** (1989) Protein synthesis by isolated castor bean mitochondria. Plant Physiol Biochem **27:** 227–234
16. **Leaver CJ, Forde BG, Dixon LK, Fox TD** (1982) Mitochondrial genes and cytoplasmically inherited variation in higher plants. *In* P Slonimsky, P Borst, G Attardi, eds, Mitochondrial Genes. Cold Spring Harbor Laboratory, New York, pp 476–484
17. **Lloyd D** (1974) The Mitochondria of Microorganisms. Academic Press, New York
18. **Maeshima M, Hattori T, Asahi T** (1987) Purification of complexes II and IV from plant mitochondria. Methods Enzymol **148:** 491–501
19. **McCarthy AD, Walker JM, Tipton KF** (1980) Purification of glutamate dehydrogenase from ox brain and liver. Biochem J **191:** 605–611
20. **Mocquot B, Pradet A, Litvak S** (1977) DNA synthesis and anoxia in rice coleoptiles. Plant Sci Lett **9:** 365–371
21. **Mocquot B, Prat C, Mouches C, Pradet A** (1981) Effect of anoxia on energy charge and protein synthesis in rice embryo. Plant Physiol **68:** 636–640
22. **Nebiolo CM, White EM** (1985) Corn mitochondrial protein synthesis in response to heat shock. Plant Physiol **79:** 1129–1132
23. **Rej R, Horder M** (1983) Aspartate aminotransferase. Methods Enzymatic Anal **3:** 416–433
24. **Ricard B, Pradet A** (1989) Anaerobic protein synthesis in different organs of germinating rice seeds. Plant Physiol Biochem **27:** 761–768
25. **Saglio PH, Drew MC, Pradet A** (1988) Metabolic acclimation to anoxia induced by low (2–4 kPa partial pressure) oxygen pretreatment (hypoxia) in root tips of *Zea mays.* Plant Physiol **86:** 61–66
26. **Saint-Ges V, Roby C, Bligny R, Pradet A, Douce R** (1991) Kinetic studies of the variations of cytoplasmic pH, nucleotide-triphosphates (31P NMR) and lactate during normoxic and anoxic transitions in maize root tips. Eur J Biochem **200:** 477–482
27. **Shibasaka M, Tsuji H** (1988) Cytochromes of mitochondria from rice seedlings germinated under water and their changes during air adaptation. Plant Cell Physiol **29:** 629–635
28. **Tassi F, Restivo FM, Ferrari C, Puglisi PP** (1983) Erythromycin as a tool for discriminating *in vivo* between mitochondrial and chloroplastic protein synthesis in *Nicotiana sylvestris.* Plant Sci Lett **29:** 215–225
29. **Vartapetian BB, Andreeva IN, Nuritdinov N** (1978) Plant cells under oxygen stress. *In* DD Hook, RMM Crawford, eds, Plant Life in Anaerobic Environments. Ann Arbor Science Publishers Inc., Ann Arbor, MI, pp 13-88
30. **Vartapetian BB, Maslov AI, Andreeva IN** (1975) Cytochromes and respiratory activity of mitochondria in anaerobically grown rice coleoptiles. Plant Sci Lett **4:** 1–8