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The mitochondrial respiratory chain of *Ustilago maydis*

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Abstract

Ustilago maydis mitochondria contain the four classical components of the electron transport chain (complexes I, II, III, and IV), a glycerol phosphate dehydrogenase, and two alternative elements: an external rotenone-insensitive flavone-sensitive NADH dehydrogenase (NDH-2) and an alternative oxidase (AOX). The external NDH-2 contributes as much as complex I to the NADH-dependent respiratory activity, and is not modulated by Ca^{2+}, a regulatory mechanism described for plant NDH-2, and presumed to be a unique characteristic of the external isozyme. The AOX accounts for the 20% residual respiratory activity after inhibition of complex IV by cyanide. This residual activity depends on growth conditions, since cells grown in the presence of cyanide or antimycin A increase its proportion to about 75% of the uninhibited rate. The effect of AMP, pyruvate and DTT on AOX was studied. The activity of AOX in *U. maydis* cells was sensitive to AMP but not to pyruvate, which agrees with the regulatory characteristics of a fungal AOX. Interestingly, the presence of DTT during cell permeabilisation protected the enzyme against inactivation.

The pathways of quinone reduction and quinol oxidation lack an additive behavior. This is consistent with the competition of the respiratory components of each pathway for the quinol/quinone pool.

Keywords: Alternative NADH dehydrogenase; Alternative oxidase; Basidiomycete; Digitonin; Electron transport chain; Salicylhydroxamic acid; Permeabilisation; *Ustilago maydis*

1. Introduction

Ustilago maydis is the causal agent of corn smut; infection by this organism produces galls in the fruits of its host plant, filled with teliospores. In many countries this fungus causes a severe damage to crops [1]. In addition, *U. maydis* is related to other phytopathogens (grouped in *Tilletia* and *Ustilago* genus) which infect economically important species such as rice, sugar cane or sorghum [1,2].

For these reasons, *U. maydis* has been the subject of an intense research, especially at the level of its genetic regulation, virulence, development, and relationship with the host [1,3], such that it is considered a model phytopathogen [1–3]. However, the bioenergetics and intermediary metabolism of *U. maydis* are far from understood, and this problem is shared with the great majority of the basidiomycetous species.

One important characteristic of plant and some fungal mitochondria is the presence of alternative components in their respiratory chains, which branch the pathway of electron transfer and are not coupled to ATP synthesis [4–6]. The most ubiquitous and prominent of these enzymes are AOX (quinol oxidase) [4] and NDH-2; the latter catalyzes the same redox reaction as complex I but does not pump protons across the mitochondrial inner membrane [5].

Abbreviations: AMP, adenosine 5-monophosphate; AOX, alternative oxidase; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; MTT, methylthiazoletetrazolium; NDH-2, alternative NADH dehydrogenase; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SHAM, salicylhydroxamic acid; TMPD, *N*, *N*, *N*′, *N*′-tetramethyl-*p*-phenylenediamine; Tris, tris(hydroximethyl) aminomethane

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However, other alternative components might be present, such as diverse dehydrogenases and quinol oxidoreductases [7–9]. Hence, the complexity of respiratory chains may vary from very simple linear chains to highly branched ones. *S. cerevisiae* illustrates the first situation, with mitochondria containing no complex I, no AOX, and two NDH-2 embedded in the inner mitochondrial membrane [4]. At the other end, plant mitochondria show a high degree of branching, with several external and internal NAD(P)H dehydrogenases in addition to complex I, and one AOX [10].

The physiological role of alternative respiratory complexes is unclear. Evidence suggests that they are involved in the adaptation of organisms to fluctuating environments. Cold stress, oxidative stress, anaerobic conditions, change in food source or in temperature [11–13] are among the factors that influence the expression or activity of alternative respiratory components. Recently, it has been addressed the question of the function of the external isoform of NDH-2. This enzyme is thought to be the main shuttle of reducing equivalents in *S. cerevisiae* [14,15].

About 30 years ago, Ziogas and Georgopoulos [16], looking for the mechanism of action of carboxin, described in *U. maydis* mitochondria a significant percentage of the respiration resistant to cyanide or antimycin A. They also reported that exogenous NADH stimulates respiratory activity, and that a percentage of this activity was resistant to rotenone. These two resistant activities were increased in the presence of chloramphenicol [17], an inhibitor of mitochondrial protein synthesis, which is known to reduce the activity of classic respiratory complexes. Together, these results suggest the presence of two alternative components, an AOX and an NDH-2.

The aim of this study was the further characterization of the mitochondrial respiratory chain of *U. maydis*, with particular emphasis on the identification and regulation of the alternative components.

2. Materials and methods

2.1. Cell culture

Strain FB_2 (a_2b_2) of wild-type *U. maydis* was used in this study. Saprobium yeast-like monokaryotic cells were grown as previously reported [18] in YPD medium (1% yeast extract, 0.25% bactopeptone, 1% glucose), pH 4.7 at 29±2 °C, under shaking at 250 rpm. The flasks were filled to one quarter of their capacity to prevent anaerobiosis. At 48 h of culture, cells were harvested by centrifugation and washed twice with distilled water. Finally, cells were suspended in KME medium (KCl 120 mM, MOPS 20 mM, EDTA 2 mM, pH 7) in which the experiments with intact and permeabilised cells were performed. Cell density was determined by reading the absorbance at 600 nm (AU_{600}). To study the long-term effect of respiratory inhibitors on the activity of AOX, cells were grown for 24 h at 30 °C, followed by the addition of inhibitor, and harvested after further 24 h of incubation at 30 °C.

2.2. Cell permeabilisation

Plasma membrane permeabilisation was achieved by incubation of *U. maydis* cells (35 AU_{600}/ml) with 20 mg/ml digitonin, for 1–3 min at room temperature, followed by centrifugation in a microfuge. Cells were resuspended in KME medium and placed on ice. To preserve the activity of AOX, permeabilisation and resuspension of cells were carried out in the presence of pyruvate 5 mM [19] and/or DTT 1 mM [20], both activators of plant AOX, and/or AMP 5 mM [21], an activator of the fungal enzyme.

2.3. Mitochondria isolation

U. maydis cells were harvested by centrifugation, washed twice with distilled water, and resuspended in MTE buffer (mannitol 600 mM, Tris–HCl 20 mM, EDTA 1 mM, pH 7.4) at a final ratio of 5 ml/g wet weight. Subsequent steps were carried out in the same buffer at 4 °C. Cells were disrupted with glass beads (in the presence of 1 mM PMSF) and mitochondria were isolated by differential centrifugation. Briefly, cell debris was eliminated by centrifugation at 3000×*g* for 10 min. The mitochondrial pellet was obtained by spinning the 3000×*g* supernatant for 10 min at 12 000×*g*, washed once to eliminate cytosolic contamination, and resuspended with MTE buffer to a final protein concentration of 10–30 mg/ml. Bovine heart and *S. cerevisiae* mitochondria were obtained as described previously [22].

2.4. Oxygen consumption

Respiratory measurements with intact and permeabilised cells were carried out in 1.5 ml of air-saturated KME medium (pH 7) at 25 °C. Oxygen consumption was determined using a Clark-type oxygen electrode.

2.5. Native blue gel electrophoresis

Native blue gel electrophoresis was performed in a 5% to 14% acrylamide gradient gel as described by Schägger and von Jagow [23]. NADH dehydrogenase activities were revealed by incubating the gel for 30–45 min in the dark, in 50 ml of Tris–HCl 20 mM, pH 7.4, with NADH 50 μM and MTT 50 μM. MTT changes its color when reduced, from yellow to violet, and precipitates at the point where the redox reaction occurs.

2.6. Immunoblot

Electrophoresis was carried out with 50 μg of mitochondrial protein, under denaturing and reducing conditions in

10% acrylamide gel. Electrotransfer to PVDF membrane was performed at 100 V for 1 h. Rabbit antiserum against *Clamydomonas reindhartii* AOX was used at 1000-fold dilution. Goat peroxidase-conjugates anti-rabbit IgG antibodies were obtained from Sta. Cruz Labs and used at 10,000-fold dilution. Luminol-based Chemiluminiscence ECL kit was obtained from Amersham Bioscience. Film exposure time was 30 s. The rabbit antiserum against *C. reindhartii* AOX was a gift from Dr. Diego Gonzalez-Halphen from Instituto de Fisiología Celular, UNAM.

3. Results

3.1. The classic respiratory complexes in U. maydis mitochondria

Since information on *U. maydis* mitochondria is scarce and fragmented, our first goal was to look for the presence of the classic components of the respiratory chain. A functional approach was used, based on the stimulation of respiration by specific substrates and inhibition of oxygen consumption by known inhibitors of complex I, III or IV. However, the initial experiments with isolated mitochondria gave conflicting results, essentially because we found some methodological problems with the isolation of intact and good quality mitochondria. Hence, we decided to work with digitonin-permeabilised cells. As shown in Table 1, addition of CCCP to digitonin-permeabilised cells did not affect their respiratory activity, suggesting the presence of uncoupled mitochondria (H^+ freely flows across the inner membrane). Nevertheless, the mitochondrial inner membrane was impermeable to small molecules like NADH. Table 1 also shows that *U. maydis* mitochondria contain the four classical respiratory complexes. Oxygen consumption was stimulated by pyruvate-malate (complex I), succinate (complex II), and TMPD-ascorbate (complex IV). As expected, rotenone (complex I), antimycin (complex III) and cyanide (complex IV) inhibited the consumption of oxygen. Besides these enzymes, *U. maydis* also contains an active mitochondrial glycerol 3-phosphate dehydrogenase (Table 1). It is worth mentioning that summation of individual respiratory rates obtained with succinate, pyruvate+malate, or exogenous NADH (130 ng AO min^{-1} $AU_{600}{}^{-1}$) is greater than the respiratory rate observed when these substrates are together (Table 1).

Table 1
Oxygen consumption by intact and digitonin-permeabilised *U. maydis* cells

Substrate	Respiratory activity (ng AO/min/AU_{600})	*N*
Intact cells	86±9	7
+KCN (1 mM)	18±8	7
+Antimycin (10 μM)	22±11	3
+Rotenone (15 μM)	40±5	3
Digitonin-permeabilised cells (−AMP −DTT)		
NADH (0.1 mM)	42±7	7
+Ca^{2+} (1 mM)	34±4	4
+ADP (50 nmol)	45±5	5
+CCCP (5 μM)	38±3	4
+Rotenone (3 μM)	44±8	6
+Antimycin (4 μM)	1±1	3
NADPH (0.5 mM)	3±1	3
Succinate (5 mM)	50±9	6
+Antimycin (4 μM)	2±1	5
Pyruvate (10 mM)+malate (10 mM)	38±5	7
+Rotenone (3 μM)	3±1	5
Glutamate (10 mM)+malate (10 mM)	40±10	3
Glycerol 3-phosphate (5 mM)	31±6	3
TMPD (1 mM)+ascorbate (5 mM)	160±12	4
+KCN (1 mM)	2±4	3
+Cytochome *c* (2 μM)	161±6	3
NADH (0.1 mM)+succinate (5 mM)+ pyruvate (10 mM)+malate (10 mM)	76±12	4
Digitonin-permeabilised cells (+AMP +DTT)		
NADH (0.1 mM)	42±4	6
+Antimycin (4 μM)	16±3	3
+KCN (1 mM)	18±4	5

Cells were permeabilised in the presence or absence of AMP (5 mM) and DTT (1 mM).
The permeabilisation procedure is described under Materials and methods. Respiratory rates represent steady state oxygen consumption. In the case of permeabilised cells, activities are reported after subtraction of the basal respiration (without mitochondrial substrate). Inhibitors were added after the steady state was reached.

3.2. The alternative oxidase in U. maydis

Since the presence of AOX and alternative NADH dehydrogenases in mitochondria is a frequent metabolic strategy used by plants and several fungi, it was not surprising to find out that in intact *U. maydis* cells and in cells permeabilised by digitonin in the presence of AMP and DTT, a significant percentage of mitochondrial respiratory activity was insensitive to rotenone, cyanide or antimycin A, pointing to the presence of additional components in the respiratory chain (Table 1). Therefore, we looked at the presence of the AOX by immunoblot (inset in Fig. 1). A single 32-kDa band was evident on the gel, with a molecular mass similar to other plant and fungal AOX, suggesting the presence of this protein in *U. maydis* mitochondria. Interestingly, the AOX activity was lost when *U. maydis* cells were permeabilised in the absence of AMP and DTT (Table 1).

Our next goal was to determine the participation of the AOX in respiration. Oxygen consumption measurements in intact cells are shown in Fig. 1. Respiratory activity of non-permeabilised *U. maydis* cells was 75–80% sensitive to cyanide (1 mM) or antimycin A (10 μM). The 20–25% residual respiratory activity was sensitive to SHAM (250 μM). Since SHAM is a good inhibitor of AOX [24], this result points to the presence of an active AOX in *U. maydis* mitochondria, and shows that mitochondrial respiration is the result of the activity of the classic pathway of quinol oxidation (cytochromes bc_1 and aa_3) and the AOX.

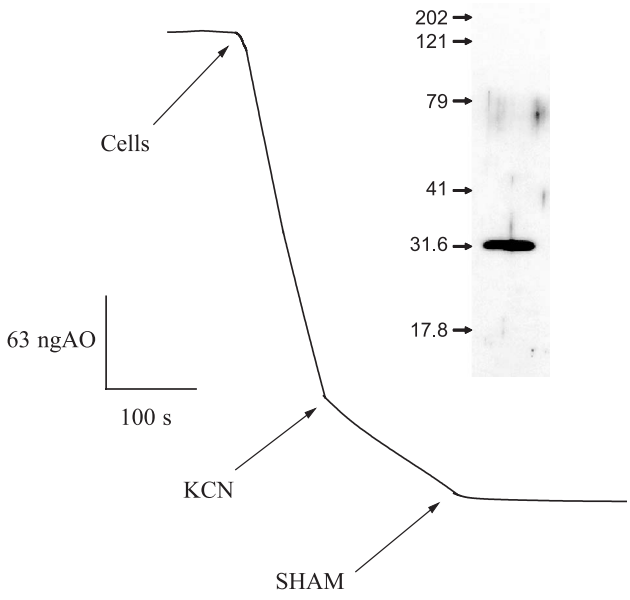

Fig. 1. Inhibition of respiratory activity in intact *U. maydis* cells by cyanide and SHAM. The arrows show the addition of inhibitor. Inset: Immunoblot of *U. maydis* mitochondrial AOX. Electrophoresis was carried out with 50 μg of mitochondrial protein, under denaturing and reducing conditions in 10% polyacrylamide gel.

Next, we concentrated on the regulation of AOX activity. It is known that many plants and microorganisms increase the expression of alternative components when they grow in the presence of oxygen free radicals [25,26] or inhibitors of the classic respiratory pathway [25,27]. In a similar way, when *U. maydis* cells were grown in the presence of cyanide (25 μM) or antimycin (10 μM), the extent of inhibition by cyanide and SHAM was modified (Fig. 2). Cyanide decreased the oxygen consumption only 5–10%, while inhibition by SHAM increased to 75%. An interesting result is that an important proportion of respiration (~15%) was not associated with mitochondrial terminal oxidase activity, since it was not inhibited by cyanide or SHAM. Since respiratory inhibitors increase the production of oxygen free radicals by mitochondria [28], it is likely that the residual oxygen consumption is the result of the activity of antioxidant enzymes related to oxygen radical handling.

AOX in plants is regulated by α-keto acids, such as pyruvate, and by the redox state of mitochondrial matrix, probably mediated by glutathione or ferredoxin [4,19,20]. In contrast, fungal alternative oxidase is regulated by purine nucleotides, but not by α-keto acids [4,21]. To study the effect of these ligands on *U. maydis* AOX, cells were permeabilised with digitonin in the absence or presence of activators of plant AOX (pyruvate and DTT) and fungal AOX (AMP). When the three ligands were present during cell permeabilisation, the activity of AOX was evident (Fig. 3). In contrast, in the absence of activators, AOX activity was lost (Table 1). Interestingly, the total respiratory rate was similar with or without an active AOX (Table 1). To further discriminate among the three putative activators, both permeabilisation of cells and measurements of *U. maydis*

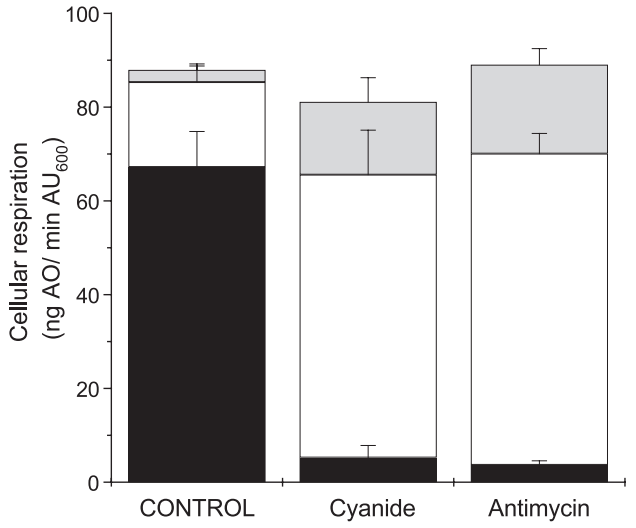

Fig. 2. Increase in AOX activity after growing *U. maydis* cells in the presence of classic pathway inhibitors. Cells were grown for 24 h in YPD at 30 °C. At this time, cyanide (25 μM) or antimycin (10 μM) was added and incubation at 30 °C continued for further 24 h. Cells were harvested and washed, and their sensitivity to inhibitor evaluated. Black, respiratory activity sensitive to cyanide (1 mM); white, respiratory activity insensitive to cyanide, but sensitive to SHAM (250 μM); gray, oxygen consumption insensitive to both inhibitors. Results from five independent experiments are expressed as the mean±standard error.

AOX activity were carried out in the presence of either pyruvate, AMP or DTT (Fig. 3). In agreement with the sensitivity of fungal alternative oxidase to activators, *U. maydis* AOX activity was lost in the presence of pyruvate but it was retained when cells were permeabilised in the presence of AMP. However, it was surprising to find out that DTT protected the enzyme, even though the only putative AOX

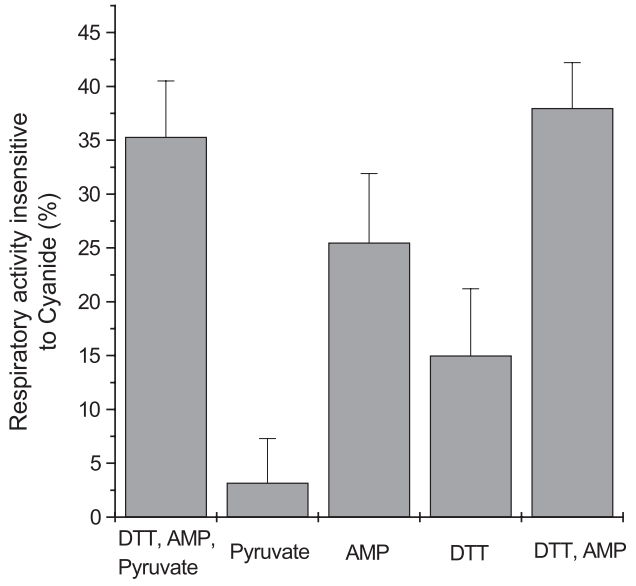

Fig. 3. Effect of AMP, pyruvate and DTT during cell permeabilisation on *U. maydis* AOX activity. Cells were incubated for 1–3 min with digitonin (20 mg/ml), either in the presence or absence of the following activators: pyruvate, 5 mM; AMP, 5 mM; DTT, 1 mM. The respiratory substrate was NADH (0.1 mM).

gene in *U. maydis* genome lacks the regulatory cysteine residues of plant AOX (http://www-genome.wi.mit.edu).

3.3. The alternative NADH dehydrogenase

As mentioned, early studies suggested the presence of an alternative NADH dehydrogenase associated with the respiratory system in *U. maydis* mitochondria, but their topology was an open subject. To address this question, we developed a protocol which takes advantage of the specificity of some inhibitors of NDH-2 and complex I, and the impermeability of inner mitochondrial membrane to small molecules. The results of these experiments are shown in Fig. 4. For permeabilised cells, exogenous NADH increases the rate of oxygen consumption, and this activity was inhibited by flavone, but not by rotenone (Fig. 4A and B). When mitochondrial substrates, such as pyruvate plus malate (10 mM each), were added to the cellular suspension, an increase in respiratory activity was evident. This respiration, in contrast to the one induced by exogenous NADH, was inhibited by rotenone but not by flavone (Fig. 4C and D). These results indicate that *U. maydis* mitochondria have two types of NADH dehydrogenases: the alternative NDH-2, facing the cytosolic side of the inner mitochondrial membrane and sensitive to flavone; and the internal rotenone-sensitive NADH dehydrogenase or complex I. This result also indicates that digitonin permeabilised the plasma membrane, leaving intact or disturbing in less proportion other intracellular compartments, such as mitochondria. In contrast to the plant alternative NADH dehydrogenase [30], the respiratory activity with exogenous NADH was not stimulated by Ca^{2+} (Table 1).

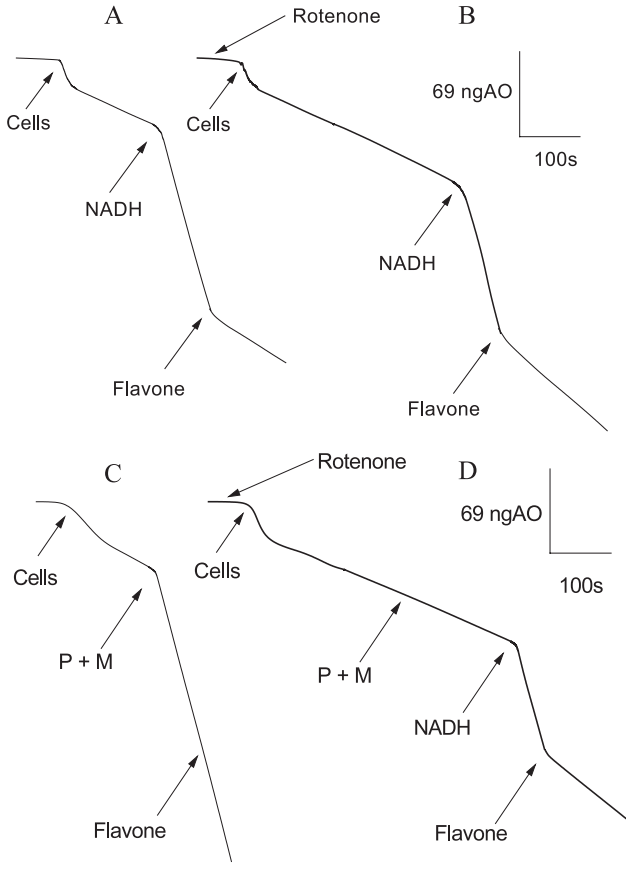

Fig. 4. Effect of rotenone and flavone on internal and exogenous NADH consumption by permeabilised *U. maydis* cells. Respiratory activity stimulated by exogenous NADH (0.1 mM) in the absence (A) or presence (B) of rotenone. Respiratory activity stimulated by pyruvate (10 mM) plus malate (10 mM) in the absence (C) or presence (D) of rotenone. Additions are indicated by arrows. Flavone (250 μM); P+M, pyruvate plus malate (10 mM each); rotenone (3 μM). Cells were permeabilised by digitonin in the absence of AMP, DTT or pyruvate. The cell density used was 3 UA_{600}.

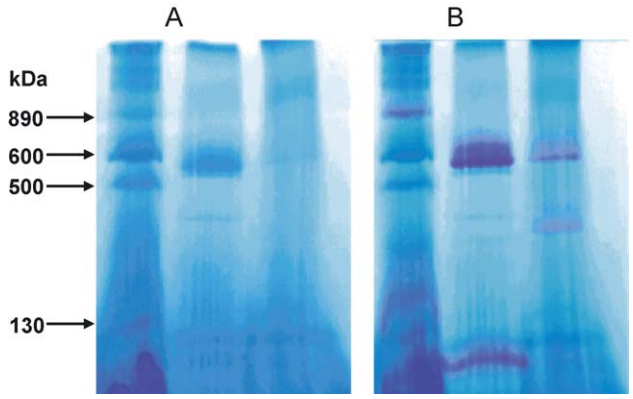

Fig. 5. Native blue gel electrophoresis of *U. maydis* mitochondria. Before (A) and after (B) incubation with NADH and MTT. To detect the presence of NADH dehydrogenase activities, the gel was incubated for 30–45 min in Tris–HCl 20 mM, pH 7.4, with 50 μM NADH and 50 μM MTT. Lanes correspond, from left to right, to bovine heart, *U. maydis* and *S. cerevisiae* mitochondria. Molecular weight standards are derived from respiratory complexes of bovine heart mitochondria; our electrophoretic pattern and the pattern shown by Schägger and von Jagow [23] are in close agreement. Complex I, 890 kDa; complex II, 130 kDa; complex III, 500 kDa and complex V, 600 kDa.

Native blue activity gel electrophoresis was important to reinforce the conclusion on the presence of two mitochondrial NADH dehydrogenases. To compare the activity pattern, two controls were included: bovine heart mitochondria, with only complex I present, and *S. cerevisiae* mitochondria, with two external and one internal NDH-2, but no complex I [4,14]. As shown in Fig. 5, bovine heart mitochondria display a single high molecular mass activity band, corresponding with the electrophoretic mobility of complex I [23]. In *S. cerevisiae* mitochondria two activity bands were detected, one of high and the other of low molecular mass. Since *S. cerevisiae* lacks complex I, our data suggest that the NDH-2s in this organism associate to produce supramolecular complexes, a result that has been reported previously [31]. The low molecular mass activity band may represent the minimal activity unit of NDH-2.

In *U. maydis* mitochondria three activity bands appeared. Similar to *Neurospora crassa* complex I, one band showed a molecular mass around 650 kDa. The other band of high molecular mass (580 kDa) probably represents a supramolecular complex of the external NDH-2(s), while the low molecular mass band (approximately 84 kDa) most likely

corresponds to the minimal activity unit of NDH-2, presumably a monomer.

4. Discussion

Very few studies on the energetics or intermediary metabolism have been done with basidiomycetous fungi, even though these organisms are important because they cause several plant diseases. Our study was directed to elucidate the components of the respiratory chain of *U. maydis*, a model fungal phytopathogen responsible of corn smut. The chain is composed of the four classic components, complexes I–IV (evidenced by specific substrate consumption and inhibitor sensitivity), a glycerol 3-phosphate dehydrogenase, and two alternative elements: AOX and the external isoform of NDH-2.

AOX accounts for the 20% residual respiratory activity in *U. maydis* cells after inhibition of the classic pathway of quinol oxidation by antimycin A or cyanide. However, this value can change as a function of the environmental conditions. For example, a characteristic feature of organisms possessing alternative oxidase is that when they grow in the presence of inhibitors of the classic pathway, the expression of the alternative components is increased [25,27]. A similar pattern was observed in *U. maydis*; cells grown in the presence of cyanide or antimycin A showed a four- to fivefold increase in the activity of AOX, while the activity of the classic pathway was decreased five- to sixfold. Since respiration in these cells is partially uncoupled to the synthesis of ATP, it opens some basic questions on the biology of *U. maydis*.

Plant AOX is regulated by α-keto acids (such as pyruvate) and the mitochondrial redox state. The modulation by α-keto acids is the result of the covalent interaction of pyruvate (in the form of thiohemiacetal) with a highly conserved cysteine residue [10,19,20]. The reduction of this cysteine residue by DTT or β-mercaptoethanol results in the activation of AOX. In contrast, fungal AOX does not have this important cysteine residue [4,21], in agreement with the lack of regulation of the fungal enzyme by pyruvate and reducing agents on this enzyme. As expected, the results showed that *U. maydis* enzyme followed the fungal behavior: its activity was stimulated by AMP and pyruvate had no effect during cell permeabilisation. A surprising result was the preservation of AOX activity by DTT. This effect was specific for DTT, since β-mercaptoethanol did not protect the enzyme during cell permeabilisation. It is likely that activation of plant AOX and protection of *U. maydis* AOX activity by DTT follow different mechanisms, since the single putative AOX gene in *U. maydis* lacks the regulatory cysteine residues found in plant AOX.

It has been shown that the responses of classic and alternative quinol oxidizing pathways are interdependent, in such a way that inhibition of one pathway results in activation of the other [29,32,33]. In *U. maydis* mitochondria we found the same phenomenon. Respiration of permeabilised cells with or without an active AOX was nearly the same, suggesting that in the presence of an active AOX the activity of the cytochrome pathway decreases to some extent, resulting in no change in total oxygen consumption. Therefore, when there is no AOX activity, quinol is oxidized by the classic pathway, but when AOX becomes active, both pathways compete for quinol, bringing about a decrease in the activity of the classic pathway.

Two mitochondrial NADH dehydrogenase activities were detected in *U. maydis* mitochondria: (a) one sensitive to flavone, facing the cytosolic side of the inner mitochondrial membrane, showing the presence of at least one external alternative NADH dehydrogenase, and (b) an internal, rotenone-sensitive dehydrogenase, indicating the presence of complex I. As with the classic and alternative quinol oxidizing pathways, the quinone reducing pathways (each one composed by a dehydrogenase) do not present an additive behavior. The coexistence of several types of NADH dehydrogenases is a common strategy of microorganisms and plants. In *S. cerevisiae* mitochondria one internal and two external alternative dehydrogenases are involved in NADH-dependent respiration, but complex I is absent [4]. On the other hand, up to two internal and two external alternative dehydrogenases, in addition to complex I [10,34], are responsible for the respiratory activity of plant mitochondria. Interestingly, *Yarrowia lipolytica* mitochondria [35] have the same distribution of dehydrogenases as *U. maydis* mitochondria. The biological value of these apparently redundant mechanisms of NADH-quinone electron transfer is not fully understood.

The sequences of some NADH dehydrogenases show a Ca^{2+} binding EF hand motif [5]. In fact, the alternative NADH dehydrogenase of plants is activated by Ca^{2+} and, depending on the isoform, calcium requirements are different among them [30]. The external enzyme in *N. crassa* mitochondria contains a putative Ca^{2+} binding motif, and it is presumed to be Ca^{2+}-sensitive [36]. However, some of these results must be taken with care, since activation by calcium was observed on mitochondrial functions, but not on purified enzyme. Exogenous NADH-dependent respiratory activity in *U. maydis* permeabilised cells is not sensitive to Ca^{2+}, implying two possibilities: (1) NDH-2 is not activated by Ca^{2+}, or (2) NDH-2 is activated by Ca^{2+}, but has a very low control flux coefficient, which means that even when its activity can be decreased or increased two or three times, there is not a parallel change in respiratory flux.

The special topology of the external alternative NADH dehydrogenase has been taken as an indication of the role of this enzyme in cellular metabolism. Mechanisms of transport of redox equivalents from cytosol to mitochondria are different in yeast and mammalian cells. Aspartate-malate shuttle is absent in fungi (those studies were made in ascomycetous fungi). However, several new shuttles arose in yeast cells to account for the lack of aspartate-malate shuttle, like the acetaldehyde-ethanol, malate-oxaloacetate, and the

external alternative NADH dehydrogenase. The relative importance of these shuttles depends on the metabolic state of the cell, and probably varies from one organism to another. Studies made in *S. cerevisiae* by Bakker et al. [14] and Luttik et al. [15] indicate the important role of the external alternative NADH dehydrogenase in the physiology of this yeast. Our experiments revealed that in addition to the external NADH dehydrogenase, *U. maydis* contains the glycerol 3-phosphate dehydrogenase shuttle for transport of redox equivalents into mitochondria.

A recent breakthrough in the field of basidiomycete fungi is the sequencing and release of the *U. maydis* genome by The Whitehead Institute in collaboration with Bayer [37]. With this information at hand, a BLAST analysis was performed on the genome sequence of *U. maydis*, using the amino acid sequence of the *S. cerevisiae* internal NADH dehydrogenase (NDI1) [38]. Three genes coding for proteins sharing high identity (33–49%) and low *E* values (2×10^{-94}–1×10^{-48}) were identified. In addition, it is predicted that these proteins contain a mitochondrial target sequence [39] and the two putative adenine nucleotide binding motifs characteristic of this protein family. After removal of the mitochondrial target sequence, the molecular masses of the mature proteins were calculated as 62.7, 69.4, and 58 kDa. Since these values are approximately the sizes of the molecular weights obtained by native blue electrophoresis, this result suggests that the minimal active unit of the NADH dehydrogenase is the monomer. Work is in progress to establish which one of the putative NADH dehydrogenase genes is expressed in *U. maydis* yeast cells growing in YPD medium.

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