Organization and regulation of the mitochondrial oxidative pathway in *Mucor rouxii*

Ruben Salcedo-Hernandez,¹ Edgardo Escamilla³ and José Ruiz-Herrera^{1,2}

Author for correspondence: José Ruiz-Herrera. Tel: +52 462 51600. Fax: +52 462 51282.

^{1,2}Departamento de Ingeniería Genética (Unidad Irapuato),¹ and Departamento de Genética y Biología Molecular (Unidad México)², Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 629, Irapuato, Gto. Mexico

³ Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico

Mitochondria from spores, anaerobic yeasts and aerobic mycelium of *Mucor rouxii* were isolated and the components of the respiratory pathways were analysed. Our data suggest the presence in these mitochondria of three different NADH dehydrogenases, three *b*- and two *c*-type cytochromes, plus three different terminal oxidases: cytochrome *c* oxidase, an incompletely characterized cytochrome which, by its spectral characteristics, behaves as a cytochrome *o*, and a salicylhydroxamic-acid-sensitive oxidase. The composition of the respiratory chain, as well as the relative proportions of cytochromes and oxidases in the mitochondria, were distinct at each developmental stage. Our data suggest that the development of the respiratory pathways during differentiation is mainly regulated by the oxygen tension. Besides this role, oxygen also influences the relative number of mitochondria in the cells and their ultrastructural organization.

Keywords: *Mucor rouxii*, respiratory pathway, mitochondria, cytochromes

INTRODUCTION

Several *Mucor* species are dimorphic fungi with the capacity to grow as mycelial or yeast-like forms, depending on the environmental conditions (Bartnicki-García, 1963). Perhaps the most important factor which regulates the morphogenetic behaviour of these fungi is the oxygen tension. Therefore, it is not surprising that substantial work has been directed to the study of the correlation existing between the respiratory activity and dimorphism. It has been observed that yeast monomorphic mutants of *Mucor bacilliformis* are respiratory deficient (Storck & Morrill, 1971; Ruiz-Herrera *et al.*, 1983), and that some respiratory or protein-synthesis inhibitors negatively affect both the formation of components of the respiratory pathway and mycelial growth (Rogers *et al.*, 1974; Zorzopulos *et al.*, 1973). Nevertheless, the existence of species, such as *Mucor rouxii*, which grow as a mycelium in anaerobic conditions (Bartnicki-García & Nickerson, 1962b), and the existence of a respiratory-competent mutant of *Mucor racemosus* (Borgia *et al.*, 1985), suggested that the relationship between oxygen and morphogenesis is not straightforward.

Recently, we have elaborated the hypothesis that a combination of redox potential and catabolite repression may trigger the response of specific morphogenetic-linked sensors associated with, but distinct from, members of the respiratory chain (Salcedo-Hernández & Ruiz-Herrera, 1993).

We have described that the spore of *M. rouxii* displays a branched respiratory pathway utilizing as terminal oxidases the normal cyanide-sensitive cytochrome oxidase and a SHAM-sensitive component. This latter component disappears when spores germinate under aerobic conditions, but remains active when they germinate in anaerobiosis. A third oxidase, cytochrome *o*, was detected at all growth stages (Cano-Canchola *et al.*, 1988). Here we describe experiments devoted to a better characterization of the respiratory pathway present in spores, anaerobic yeasts and aerobic mycelium of *M. rouxii*, by use of isolated mitochondria from all these stages. Our data suggest that the respiratory chain is more complex than anticipated, with the participation of several NADH dehydrogenases and different cytochrome combinations, plus the already described three terminal oxidases.

METHODS

Organisms and culture conditions. *Mucor rouxii* IM80 (ATCC 24905) was used in this study. It was maintained on slants of solid YPG medium (Bartnicki-García & Nickerson, 1962a).

Abbreviations: CCCP; carbonyl cyanide *m*-chlorophenylhydrazone; SHAM, salicylhydroxamic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine.

Spores were harvested with sterile distilled water, washed by centrifugation and resuspended in sterile distilled water. Their numbers were measured with a haemocytometer and kept at -20°C in 50% (w/v) glycerol. Under these conditions, they remained viable for more than one year. Liquid YPG medium was inoculated with 5×10^5 – 10^6 spores ml^{-1} and incubated under different conditions, as indicated for each experiment. Aerobiosis was provided by shaking, whereas for anaerobic growth, cells were sparged either with a mixture of CO_2/N_2 (30:70, v/v) or with a sterile N_2 stream from which traces of O_2 were removed by passage through a stainless steel tube packed with copper filings and heated at 600°C . Cells for electron microscopy were obtained in the minimal defined medium of Bartnicki-García & Nickerson (1962a). For the isolation of mitochondria from yeast-like cells in some experiments, a 'self-anaerobic' method was employed (Salcedo-Hernández & Ruiz-Herrera, 1993). Spores (10^6 ml^{-1}) were inoculated into 2 litre flasks filled with YPG medium and stirred with a magnetic bar. Flasks were sealed with rubber stoppers provided with a needle to permit the exit of excess CO_2 .

Isolation of mitochondria. A method developed by G. Ortiz (personal communication) was employed. Glass beads (1 mm in diameter for mycelium, or 0.5 mm in diameter for spores) were used to half-fill Duran flasks. Mycelium (10 g wet wt per 100 ml) or spores (1.5×10^9 ml^{-1}), resuspended in 10 mM Tris/HCl buffer, pH 7.5, containing 2 mM EDTA, 1 mM MgCl_2 , 0.7 M sucrose and 1.1 M glycerol (medium A), were added to fill the flasks, and the cells were broken for 20 s in a Braun cell homogenizer. The suspensions were centrifuged at 5000 r.p.m. in a JA20 (Beckman) rotor for 5 min, and the supernatant was recovered and centrifuged for 10 min at 12000 r.p.m. in the same rotor. Sediment was carefully removed in Tris buffer containing EDTA, MgCl_2 and sucrose, as above, but no glycerol (medium B), and the suspension was centrifuged at 3000 r.p.m. for 5 min. Mitochondria in the supernatant were sedimented by centrifugation at 10000 r.p.m. for 10 min and recovered in a minimum volume of medium B.

Mitochondria from yeast-like cells were isolated by the same procedure as used for mycelial mitochondria, but the first supernatant was centrifuged at 15000 r.p.m. for 20 min, and the sediment was resuspended in medium B. The suspension was centrifuged at 4000 r.p.m. for 5 min, and finally the mitochondrial fraction was sedimented at 12000 r.p.m. for 15 min and recovered in the same medium.

Submitochondrial particles were obtained by sonication as described by Lee & Ernster (1967).

Determination of respiratory activity. Cells were recovered by centrifugation, washed once with sterile distilled water and resuspended in a small volume of ice-cold fresh YPG medium. Mitochondrial aliquots, resuspended in medium B, were mixed with a solution containing 120 mM KCl, 5 mM MOPS/ K_2HPO_4 , 5 mM MgCl_2 , 2 mM EDTA, and the corresponding substrates. Respiration was measured with a Clark oxygen electrode (Yellow Springs Instruments) in a 1.2 ml volume cell. Temperature was maintained at 28°C .

Determination of difference spectra of cytochromes. Mitochondria were resuspended in 0.5 M Tris/phosphate buffer, pH 7.2, containing 25% (v/v) glycerol. Some samples were reduced with ascorbate, solid sodium dithionite or 2 mM NADH, whereas others remained oxidized. After 2 or 30 min, samples were frozen with liquid N_2 in a Dewar flask, and their differential spectra (reduced minus oxidized) were measured with an SLM-Aminco DW2000 spectrophotometer. Spectral values were corrected by baseline subtraction.

Baselines were determined running oxidized minus oxidized and reduced minus reduced spectra. Each spectrum corresponds to the mean of five scans, which were further electronically smoothed.

Determination of pyridine haemochromogens. Pyridine haemochromogens were measured as described by Berry & Trumpower (1987). About 1 mg mitochondrial protein, resuspended in a mixture of 500 μl water, 500 μl NaOH (200 mM) in pyridine (40% by volume) and 3 μl 0.1 M $\text{K}_3\text{Fe}(\text{CN})_6$, was placed into a 1 ml cuvette and mixed. Oxidized spectrum was recorded as a baseline. Solid sodium hydrosulphite (2–3 mg) was then added, and 20 min later, spectra of the reduced pyridine haemochromogens were recorded. Absorbance at selected wavelengths (430, 540, 549, 553, 558, 570, 580, 588, 600, 610 and 620 nm) were multiplied as a vector by the inverse of the redundant matrix of the difference (reduced minus oxidized) absorption coefficients at these wavelengths to obtain the respective concentrations of each haem class.

Miscellaneous. Protein was measured with Folin's reagent after solubilization for 2 h with 1 M NaOH. Material for electron microscopy was fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, and postfixed with 2% (w/v) osmium tetroxide. Samples were dehydrated in a graded acetone series and embedded in Epon-Araldite. Silver interference-colour sections were collected over copper grids coated with Formvar and carbon, and post-stained with lead citrate. Sections were examined and photographed with a Jeol 100S transmission electron microscope operating at 80 kV. Experiments were repeated at least twice, with duplicate samples. Unless otherwise indicated, all reported quantitative data correspond to means of at least two experiments where the observed differences were below 10%.

RESULTS

Sensitivity of *in vivo* respiration to inhibitors

Sporangiospores of *M. rouxii*, as previously described (Cano-Canchola *et al.*, 1988), display a branched respiratory pathway partially sensitive to both CN^- and SHAM. This characteristic disappears upon aerobic growth (Table 1). It was noticed also that respiration of the spores and yeast-like cells was insensitive to rotenone. Rotenone sensitivity in spores is acquired only after aerobic incubation, when the SHAM-sensitive pathway is shut off (Table 1). Interestingly, uncoupled yeast cells became more sensitive to rotenone, antimycin and cyanide, but not to SHAM. This different behaviour is probably due to loss of respiratory control imposed at the coupling sites, not found in the SHAM bypass.

The respiratory pathway of spores, anaerobic yeast-like cells and aerobically grown cells differed in coupling. Spore respiration was completely resistant to oligomycin, whereas in contrast, germling and yeast respiration was partially sensitive to the inhibitor; more so in germlings than in yeast-like cells. Nevertheless, respiratory activity of all types of cells was stimulated by CCCP (Table 2). Addition of CCCP did not stimulate, nor did the presence of oligomycin inhibit, respiratory activity of yeast cells in the presence of KCN (Table 2). This result confirms that, as in other systems (Henry & Nyns, 1975), the alternative SHAM-sensitive terminal oxidase is not linked to energy conservation.

Table 1. Sensitivity of *M. rouxii* *in vivo* respiration to classical electron transport inhibitors

After the incubation times indicated, aliquots were centrifuged and cells resuspended in a small volume of YPG medium. Respiration was measured at 28 °C.

Cells and time of incubation	Inhibition of respiration (%)				
	Rotenone (8 µM)	Antimycin (4 µM)	KCN (2 mM)	SHAM (2 mM)	KCN + SHAM
Spores 0 h	0	0	0	35	100
Rounded spores 2 h in air	49	90	100	0	—
Swollen spores 4 h in air	59	100	100	0	—
Germlings 6 h in air	60	100	100	0	—
Yeast-like cells 12 h anaerobiosis					
Normal	4	40	57	22	70
Uncoupled with CCCP	23	81	82	17	92

Table 2. Sensitivity of respiration to oligomycin and CCCP

Respiration was measured as described in Methods and in Table 1. Where indicated, 5 µg oligomycin ml⁻¹, 4 µM CCCP or 2 mM KCN were added. CCCP stimulation in the presence of oligomycin was measured 5 min after oligomycin addition.

Cells	Oligomycin inhibition (%)		CCCP stimulation (-fold)		CCCP stimulation in the presence of oligomycin (-fold)
	Control	+ KCN	Control	+ KCN	
Spores	0	0	2.10	None	2.15
Swollen spores 4 h in air	46	*	1.34	*	2.3
Yeast-like cells 12 h anaerobiosis	17	0	1.94	None	2.5

* Respiration was completely inhibited by KCN (see Table 1).

Incubation of aerobic germlings in the presence of inhibitors specific for one of the respiratory coupling sites (1, 2, or 3) induced the appearance of the SHAM-sensitive pathway (Table 3). A similar phenomenon was induced by starvation of mycelial cells (not shown).

Ultrastructure of mitochondria and their isolation

The morphology of mitochondria in yeast and mycelium forms of the fungus was studied by electron microscopy of sections of whole cells. Two parameters measured in median sections illustrate the different enrichment of mitochondria in both types of cells: their number per unit area, and the percentage of section surface occupied by mitochondria (Table 4). Mitochondria were about four times more abundant per unit area in mycelial cells than in yeast cells; and the section surface covered by mito-

chondria was about seven times higher in mycelial compared to yeast cells. The reason why this ratio is higher than the previous one is that mycelial mitochondria are larger than those of yeast. Finally, it was observed that the planar mitochondrial cristae were twice as abundant in mycelial than in yeast mitochondria (Fig. 1). Mitochondria present in anaerobic mycelium were practically identical to those present in yeast cells (Table 4, Fig. 1c), and both of them were similar to those present in the spores of the fungus, as judged from published electron micrographs (Bartnicki-García *et al.*, 1968).

Characteristics of isolated mitochondria

In order to gain a better understanding of the characteristics of the respiratory pathway in *M. rouxii*, we proceeded to the isolation of mitochondria. Using the

Table 3. Induction of the alternative respiratory pathway by the addition of electron transport inhibitors

Aerobic cultures (5 h old) received the addition of either 2 mM KCN, 4 μ M antimycin or 2 μ M rotenone for 2 h, a culture without addition remaining as control. After 2 h further incubation, cells were harvested by centrifugation, washed once with distilled water, centrifuged again and resuspended in fresh YPG medium. Respiratory activity was measured as described in Methods.

Inhibitor in growth medium	Inhibition of respiration (%)		
	KCN (2 mM)	SHAM (2 mM)	KCN + SHAM (2 mM each)
None	96.5	0	—
KCN	54.5	0	98
Antimycin	0	93	100
Rotenone	70.5	—14.5*	100

* Respiratory stimulation.

technique described above, good yields of mitochondria were routinely obtained from young mycelium. Conservation of function in the organelles was assessed by their respiratory control, using as substrate the couple malate/glutamate (a value which was always above 2.0), and by their appearance under the electron microscope (Fig. 2a).

Mitochondrial preparations from spores and anaerobic yeasts gave lower yields because of their low number per cell (see above). These mitochondria did not exhibit measurable respiratory control. In the case of spores, this result agrees with the *in vivo* data. For the yeast cells, the possibility that mitochondrial damage was partially responsible for this effect cannot be ruled out. Nevertheless, the microscopic appearance of the mitochondria was normal (Fig. 2b).

Functional characterization of mitochondria

Mitochondria from all types of cells actively oxidized added NADH at rates 2–3 times higher than substrates which produce endogenous NADH, such as malate. Oxidation of exogenous NADH was insensitive to rotenone, as was the case for succinate, whereas glutamate oxidation was partially sensitive to rotenone addition (Table 5 and 6). It is interesting that respiration of mitochondria isolated from starved mycelium was partially sensitive to SHAM, indicating the development of the alternative respiratory pathway under these conditions. SHAM inhibited the oxidation of both endogenous NADH and succinate. Oxidation of NADH by submitochondrial particles prepared by sonication of mycelial mitochondria (see Methods) was partially sensitive to rotenone (Table 5C). As expected, succinate oxidation by these particles was resistant to the drug.

Respiratory activity of yeast mitochondria was partially sensitive to rotenone. Also SHAM or KCN were unable to inhibit respiration completely, when either NADH or glutamate was used as substrate. Full inhibition was obtained only when both inhibitors were present (Table 6).

Quantification of haemochromes

Since the absorption coefficients of *M. rouxii* cytochromes are unknown, in order to obtain a quantitative comparison of cytochromes present in mitochondria isolated from yeast and mycelium, we turned to a method which is based on the spectral analysis of the pyridine-haemochromes (Berry & Trumpower, 1987). Mycelial mitochondria contain about equal levels of cytochromes of the *a* and *b* types, and slightly higher amounts of cytochromes of the *c* type (Table 7). Yeast mitochondria, on the other hand, contained about the same amounts of cytochromes of the *b* type as compared with mycelial mitochondria, but levels of cytochromes of *c* type, and more noticeably of the *a* type, were very low (Table 7).

Table 4. Morphometric analysis of mitochondria *in situ*

The results are means of the no. of determinations indicated in column 2 $\pm$ SE.

Culture conditions	Sections analysed	Mitochondria			
		No. per 100 μ m ²	Length (μ m)	No. of cristae*	Percentage of section surface
Aerobic germlings	5	30.2 $\pm$ 3.8	1.7 $\pm$ 0.6	13 $\pm$ 4	14.3 $\pm$ 2.5
Anaerobic yeast cells	4	8.4 $\pm$ 2.0	1.2 $\pm$ 0.3	7 $\pm$ 2	2.2 $\pm$ 0.7
Anaerobic germlings	6	9.8 $\pm$ 2.3	1.1 $\pm$ 0.4	7 $\pm$ 1.5	5.5 $\pm$ 1.2

* Per mitochondrion. Numbers of cristae were determined only in mitochondria with typical morphology.

Fig. 1. Structure of mitochondria in yeast and mycelial cells of *M. rouxii*. Sections prepared as described in Methods were observed under the electron microscope. (a), Aerobic mycelium; (b), anaerobic yeast; (c), anaerobic mycelium. Bar, 0.5 μ m.

Spectral analysis of cytochromes

Spectra of chemically reduced (sodium hydrosulphite) minus air-oxidized mitochondrial samples obtained from spores, yeast and mycelium are shown in Fig. 3. Several

Fig. 2. Electron microscopic appearance of isolated mitochondria from mycelial and yeast cells. (a), Mycelial mitochondria; (b), yeast mitochondria. Bar, 0.5 μ m.

important differences which confirm previous data and provide new information can be summarized. The differences in the levels of cytochromes aa_3 among the different mitochondria are noticeable. These differences can be judged not only from the α bands, but mainly from the relative absorbance values of the Soret peaks at 443 nm (characteristic of cytochrome aa_3) and 423–427 nm (corresponding to cytochromes b and c). Spectra obtained from spore or yeast mitochondria reduced with exogenous NADH were indistinguishable from those obtained using hydrosulphite as reductant (Fig. 4). This

Table 5. Respiratory sensitivity of mitochondria and submitochondrial particles from mycelium to some electron transport inhibitors

Mitochondria were isolated from 7-h-old mycelium as described in Methods. Their respiratory activity towards three different substrates, 0.1 mM NADH, 5 mM glutamate or 5 mM succinate, was measured in the absence or presence of 8 μ M rotenone, 1 mM SHAM or 2 mM KCN. Experiment A, mitochondria isolated from fresh mycelium; experiment B, mitochondria isolated from mycelium starved for 1 h in 0.9% NaCl, a condition which induces the appearance of the alternative respiratory pathway; experiment C, submitochondrial particles were prepared as described in Methods. ND, Not determined.

Expt	Inhibitor	Inhibition of respiration (%) with:		
		NADH	Glutamate	Succinate
A	Rotenone	0	24	0
	KCN	90	93	100
B	Rotenone	7	46	0
	SHAM	54	ND	54
	SHAM + KCN	93	97	96
C	Rotenone	55	ND	0

Table 6. Sensitivity of respiration of mitochondria from anaerobic yeast-like cells towards several inhibitors

Mitochondria were isolated from 12-h-old yeast-like cells, and their respiratory activity with different substrates and inhibitors was measured as described in Methods and in Table 5.

Inhibitor	Inhibition of respiration (%) with:		
	NADH	Glutamate	Succinate
Rotenone	7	38	0
Antimycin	76	29	—
SHAM	39	40	—
SHAM + KCN	100	100	100

Table 7. Determination of haemochrome levels in isolated mitochondria

Haemochromes from mitochondria were measured as described in Methods.

Haemochrome type	Concentration (nmol mg ⁻¹)	
	Mycelium	Yeast-like cells
<i>a</i>	165	23
<i>b</i>	215	269
<i>c</i>	311	63

Fig. 3. Difference spectra of isolated mitochondria. Samples were reduced with sodium hydrosulphite and their spectra at liquid N₂ temperature were obtained versus an air-oxidized sample. A,D, Spore mitochondria; B,E, mitochondria from aerobic mycelium; C,F, yeast mitochondria. Note difference in absorbance scale between A,B,C and D,E,F. **Fig. 4.** Difference spectra of isolated mitochondria. Samples were prepared as for Fig. 3, but mitochondria were reduced for 30 min at room temperature with NADH before running the spectra at liquid N₂ temperature. A,C, Spore mitochondria; B,D, yeast mitochondria. Note the difference in absorbance scale between A,C and B,D.

result suggests that all the identified cytochromes are part of the electron transport chain of the mitochondria.

There appears to exist a complex family of *b*-type cytochromes, and not only one as previously described (Cano-Canchola *et al.*, 1988). Two *b* cytochromes, *b*₅₆₁ and *b*₅₅₂, are clearly separated in the spectra of spore and mycelial mitochondria (Figs 3D, E, F and 4), whereas yeast mitochondria contain mainly *b*₅₅₂ and small amounts

Fig. 5. Second derivative of mitochondrial spectra. A,B,C, Mitochondria reduced with sodium hydrosulphite; D,E,F, mitochondria reduced with ascorbate/TMPD. A,D, Spore mitochondria; B,E, mitochondria from aerobic mycelium; C,F, yeast mitochondria.

Fig. 6. Difference spectra of spore mitochondria reduced with NADH or ascorbate. A, Reduced for 30 min at room temperature with NADH; B, as A, but reduced for 10 min; C, reduced at room temperature with ascorbate for 20 min.

of a different *b* cytochrome, b_{558} (Fig. 5C). The reason behind considering the cytochrome with the α band maximum at 552 nm as a *b*-type, and not a *c* cytochrome,

was the low level of *c*-type haemochrome present in yeast mitochondria where *b* haemochrome is well represented (Table 7). In the yeast mitochondria, b_{561} appears only as a shoulder, and it is only barely visible in the second derivative (Figs 3F and 5C). Cytochrome c_{547} was fairly reduced by ascorbate/TMPD in mitochondria from all stages, whereas cytochrome b_{562} was only partially reduced under these conditions (Fig. 5D, E, F).

We found that there are two, instead of one, *c*-type cytochromes: cytochrome c_{547} and cytochrome c_{543} . The first one stands out clearly only in mycelial mitochondria; in spore and yeast mitochondria it appears only as a small shoulder (Fig. 5A, B, C) when they were reduced with hydrosulphite. Cytochrome c_{543} was detected only in aerobic mycelium (Fig. 5A, B, C).

Spectra of mitochondria, mainly from the spores, but also from the yeast cells, showed two broad peaks at 471 and 506 nm (Figs 3A and 4) whose relative absorbance levels varied from one experiment to another. These peaks do not correspond to any typical respiratory component. Bendall & Bonner (1971) observed similar spectral components in mitochondria from skunk cabbage (*Symplocarpus foetidus*) displaying the alternative (SHAM-sensitive) electron-transport pathway. When we used the physiological reductant NADH instead of hydrosulphite, these two non-identified peaks became apparent only after prolonged incubation times (30 min, Figs 4 and 6). A similar behaviour was obtained when ascorbate was used as reductant (Fig. 6).

DISCUSSION

The data presented above indicate that the mitochondria from *M. rouxii* are extremely plastic in their organization, and that oxygen plays a paramount role in regulating not only the composition of their respiratory pathway, but also the rate of their synthesis (compare their relative numbers in anaerobic yeasts and aerobic mycelium) and their structural organization. A similar role for oxygen has been demonstrated in the yeast *Saccharomyces cerevisiae* (Zitomer & Lowry, 1992).

According to our results, the mitochondria from spores of *M. rouxii* are oxidatively competent, but are unable to carry out ATP synthesis. When spores initiate the first stages of germination, their mitochondria become competent in ATP synthesis, as judged by their development of sensitivity towards oligomycin. At the same time, their respiratory pathway undergoes a drastic change, becoming very similar to that in animal cells (Lehninger, 1964; Hatefi, 1985).

It is interesting that respiration of spore mitochondria is completely resistant to rotenone, a classical inhibitor of site 1, indicating that they contain a rotenone-resistant NADH dehydrogenase. The fact that these mitochondria also oxidize internally generated NADH suggests that this enzyme is probably different to the rotenone-resistant enzyme which is oriented towards the cytoplasm, and which is involved in the oxidation of extramitochondrial NADH. The latter NADH dehydrogenase was detected

in yeast and mycelial mitochondria of *M. rouxii*, and appears similar to that described in other fungal species (Bruinenberg *et al.*, 1985; Schwitzgubel & Palmer, 1982). The presence of this enzyme explains why mycelial respiration was not completely sensitive to the drug. The existence of an intramitochondrial rotenone-resistant NADH dehydrogenase is supported by the observation that NADH dehydrogenase measured in submitochondrial particles (which supposedly are inside-out) is only partially sensitive to the drug. The fact that respiration of both yeast and mycelial mitochondria was only partially sensitive to rotenone suggests that appearance of the normal NADH dehydrogenase occurs during spore germination under either aerobic or anaerobic conditions. Respiration data obtained *in vivo* with coupled or uncoupled yeast cells suggest that these cells contain lower amounts of the sensitive enzyme than mycelium.

The potential of spore mitochondria to develop into normal mitochondria is regulated by the environmental conditions for growth. Under aerobiosis, there occurs an increase in the relative proportion of cytochromes of the *c* and *a* type; whereas under anaerobic conditions, levels of cytochrome *b*₅₆₁ decrease to minimal values, and there appears a new *b*-type cytochrome with a maximum of its α band at 558 nm. The rest of the cytochromes remain in about the same relative proportions as in the spore mitochondria.

Mitochondria of either spores, anaerobic yeasts or aerobic mycelium contain three oxidases, although in different proportions. These oxidases are the normal cytochrome *c*-oxidase, a still non-characterized component which, by its spectral characteristics, we have tentatively considered as a cytochrome '*o*', and the SHAM-sensitive oxidase (Cano-Canchola *et al.*, 1988). Cytochrome oxidase is predominant in mitochondria from aerobic mycelium, whereas its levels are very low in spore and yeast mitochondria, whose main terminal oxidase is the SHAM-sensitive component. This is particularly noticeable in the mitochondria from the spore, as judged by the observation that its respiration is completely resistant to either antimycin or KCN. Disappearance of the SHAM-sensitive pathway during spore germination depends on the presence of oxygen: anaerobic yeasts conserve this alternative respiratory mechanism, whereas it can no longer be detected after a few hours of spore incubation in aerobic conditions. Its reappearance in aerobically grown cells occurs as a reaction to stress, such as incubation in the presence of respiratory inhibitors, or starving conditions. In this sense, *M. rouxii* behaves as other fungal systems (Henry & Nyns, 1975; Shepherd *et al.*, 1978; Bertrand *et al.*, 1983).

Partial sensitivity of succinate oxidation by isolated mitochondria towards SHAM suggests that the SHAM-sensitive oxidase accepts electrons from the quinone pool, and the observation that neither oligomycin nor CCCP affects SHAM-sensitive respiration of yeast cells is indicative that this pathway is not linked to oxidative phosphorylation. According to this behaviour, it may be

Fig. 7. Proposed scheme for the electron transport chain in mitochondria from *M. rouxii*. Short solid arrows represent the currently accepted electron flow in other systems; dashed lines represent hypothetical electron transfer suggested from the data here presented. FPs, Succinate dehydrogenase; FPr1, rotenone-sensitive NADH dehydrogenase; FPr2, internally oriented NADH dehydrogenase resistant to rotenone; FPr3, externally oriented NADH dehydrogenase resistant to rotenone; Q, quinone pool; Os, SHAM-sensitive terminal oxidase.

suggested that the shunt pathway serves only as a valve which discharges reducing power, as occurs in plant systems (Lambers, 1980, 1982; Siedow & Berthold, 1986).

A schematic representation of the components of the respiratory pathway identified by all the evidence described above in the mitochondria at each developmental stage of *M. rouxii* is represented in Fig. 7. Their ratios, however, as discussed above, are very different in the three stages analysed.

The characteristics of the two unusual bands detected in the difference spectra of spore and yeast mitochondria at 471 and 506 nm deserve a further comment. Their variation from one to another batch of mitochondria is indicative that they are not normal members of the mitochondrial respiratory chain, but their reduction with NADH suggests that they may play a physiological role. The kinetics of reduction of these components by NADH may be taken as evidence that they have a very low redox potential, and the position of their maxima suggests that they may contain Fe-sulphur centres as their prosthetic groups.

ACKNOWLEDGEMENTS

This work was carried out with partial support from Consejo Nacional de Ciencia y Tecnología (CONACyT), México. J.R.H. is a National Investigator México.

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Received 4 May 1993; revised 26 July 1993; accepted 25 August 1993.