Respiratory Components of *Aspergillus niger* Mitochondria

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The respiratory components of tightly coupled mitochondria from the filamentous fungus *Aspergillus niger* were studied. Cytochromes \( a + a_1 \), \( b \), and \( c + c_1 \) were identified by difference spectra. The cytochrome spectra were qualitatively similar to yeast and rat liver mitochondria. The mitochondria contained, per gram of protein, an average of 2.9 and 7.0 \( \mu \)moles of ubiquinone and nicotinamide adenine dinucleotide, respectively.

Most investigations of mitochondrial metabolism have involved the use of mitochondria extracted from animal tissues such as rat liver and beef heart. Only recently has there been an increasing interest in obtaining mitochondria from other sources such as plants and microorganisms.

Since Wiskich and Bonner (28) described the preparation of tightly coupled mitochondria from sweet potato plants, there have been numerous reports on the properties of mitochondria from many other plant sources; e.g., avocado fruit (14), potato tubers (25), corn (8), and mung bean hypocotyls (9). Although mitochondrial fractions which are capable of oxidative phosphorylation have been isolated from yeast cells (26), mitochondria with respiratory control were first described by Duell, Inoue, and Utter (4) and Ohnishi and Hagihara (21). The method of extraction consisted of digestion of the yeast cell wall by snail enzyme, followed by disruption of the resulting protoplasts and centrifugation of the mitochondria.

Recently coupled mitochondria have been extracted from the filamentous fungi *Aspergillus niger* (27) and *Neurospora crassa* (7).

The mitochondria from *N. crassa* carried out oxidative phosphorylation, but were loosely coupled and appeared damaged when examined by electron microscopy. Mitochondria from *A. niger* were tightly coupled and showed a close similarity in biochemical properties to intact yeast and mammalian mitochondria.

The present communication is a study of the respiratory components of isolated *A. niger* mitochondria.

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ing 150 ml of medium having the following composition: glucose, 25 g; NH₄NO₃, 2.5 g; KH₂PO₄, 2 g; MgSO₄·7H₂O, 0.25 g; FeSO₄·7H₂O, 0.25 g; ZnSO₄·7H₂O, 1.6 mg; CuSO₄·5H₂O, 0.23 mg; MnCl₂·4H₂O, 5 mg; CaCl₂·4H₂O, 20 mg; and distilled water to 1 liter. The pH of the medium was 4.5.

Cytochrome spectra were measured in a split-beam spectrophotometer built by M. Klingenberg (Marburg, Germany). Wavelength pairs and millimolar extinction coefficients used were: cytochromes a + a₈, 605-630 nm, 24; cytochromes c + c₁, 550-540 nm, 18; cytochrome b, 564-575 nm, 19.

Extraction and estimation of ubiquinone. Mitochondrial suspensions (2 ml, containing 5 to 10 mg of protein/ml) were extracted at 5 C with 10 ml of a mixture of methanol and ligroin (60:40). The sample was centrifuged to separate the layers, and the extraction was repeated with 3 ml of ligroin. The combined ligroin extracts were evaporated under reduced pressure in a microrotary evaporator at 30 C. Depending on the concentration of the mitochondria, 5 to 7 ml of spectrophotometrically pure ethyl alcohol was added, under a nitrogen atmosphere, to the residue in the flask.

Spectra were determined in a Unicam SP800 recording spectrophotometer fitted with an expanded-scale slave recorder. Oxidized ubiquinone was measured by difference spectra by adding a trace of solid sodium borohydride to the sample. Total ubiquinone was estimated by the method of Kroger and Klingenberg (13), by treating the ethyl alcohol extracts with a solution of FeCl₃ in methanol. The wavelength pair 280 to 289 nm and a millimolar extinction coefficient of 8.5 were used (13).

Estimation of nicotinamide adenine dinucleotide (NAD). Trichloroacetic acid extracts of mitochondrial suspensions were enzymatically estimated for endogenous NAD by use of alcohol dehydrogenase (12).

RESULTS

Before centrifugation, difference spectra of cell-free extracts showed spectral bands characteristic of cytochromes a + a₈, c + c₁, and b. The bands were more distinct and resolution improved when the isolated mitochondria were examined. The succinate plus ADP reduced [state 5, using the nomenclature of Chance and Williams (3)] minus oxidized difference spectra of the mitochondrial fraction is shown in Fig. 1. We observed absorption bands typical of the a-bands of cytochromes a + a₈ at 604 nm; of cytochromes c + c₁ at 549 nm, with a shoulder at 560 nm due to cytochrome b; and of the combined b-bands at approximately 520 nm. In the Soret region, peaks at 443 nm, representative of the G-band of cytochromes a + a₈, and at 430 nm, representative of the G-band of cytochrome b, were seen.

Addition of antimycin A to the mitochondrial suspension resulted in the oxidation of cytochromes a + a₈ and c + c₁, and the appearance of the reduced bands of cytochrome b at 560 and 430 nm. The absorption maxima of the cytochromes and the response in the redox states to antimycin A are similar to that reported for yeast mitochondria (22, 23) and rat liver mitochondria (6).

With succinate, citrate, or 2-ketoglutarate as

![Fig. 1. Difference spectra of A. niger mitochondria with succinate as substrate. Mitochondria equivalent to 1.5 mg of protein suspended in a reaction mixture containing 0.3 mM mannitol, 0.2 mM EDTA, 10 mM potassium phosphate, 5 mM KCl, and 4 mg of albumin; final pH 6.5; final volume, 0.35 ml; optical path, 0.5 cm. Solid line: The contents of both cuvettes were aerated with oxygen, and then 5 mM succinate and 0.2 mM ADP were added to the sample cuvette. Broken line: The contents of both cuvettes were aerated with oxygen, and then 1 mg of antimycin A was added to the sample cuvette.](http://jb.asm.org/)
substrates, a broad absorption band at 340 to 350 nm (not illustrated), probably due to reduced pyridine nucleotide, was observed. When reduced pyridine nucleotide itself was a substrate, spectra similar to those shown in Fig. 1 were obtained. The absorption intensity of the cytochromes increased slightly when sodium dithionite was used as a reducing agent.

Polarographic traces of oxygen uptake, showing the effect of added cytochrome c on the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and 2-ketoglutarate, are illustrated in Fig. 2.

Exogenous NADH oxidation was markedly increased by the addition of cytochrome c. The cytochrome c-stimulated NADH respiration was sensitive to antimycin A. Oxidation of succinate, citrate, and 2-ketoglutarate was stimulated, on the average, by about 0 to 20%, a result which may indicate a partial loss of cytochrome c during the extraction procedure.

Figure 2 also illustrates a result noted in some preparations with succinate, citrate, and 2-ketoglutarate as substrates; active respiration was faster on the second addition of ADP than on the first addition of ADP, resulting in an increase in respiratory control. Some plant mitochondria also show an initial lag in oxygen uptake, an effect which may be overcome by preincubation of the mitochondria with adenosine triphosphate (28, 29).

The ubiquinone content of isolated A. niger mitochondria was estimated by extraction with methanol-ligroin and measurement by difference spectra. The characteristic trough of reduced ubiquinone with a minimum at 275 nm, produced by a trace of sodium borohydride, is shown in Fig. 3. No attempt was made to purify the methanol-ligroin extracts, and it was assumed that sodium borohydride reduced only ubiquinone in the wavelength range 270 to 290 nm. In six separate determinations, approximately 70 to 80% of the total ubiquinone was extracted in the oxidized state.

Data on the respiratory components of A. niger mitochondria are presented in Table 1, which, for comparison, also summarizes data on the respiratory components of yeast mitochondria (21, 22).

**DISCUSSION**

With the exception of the yeasts, previous reports of the presence of a cytochrome spectrum in fungi similar to that of the mammalian system have been confined to studies on mycelial cultures
TABLE 1. Content of respiratory components of A. niger mitochondria

<table>
<thead>
<tr>
<th>Component</th>
<th>A. niger</th>
<th>S. carlsbergensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochromes a + a1</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>0.19</td>
<td>0.28</td>
</tr>
<tr>
<td>Cytochromes c + c1</td>
<td>0.39</td>
<td>0.65</td>
</tr>
<tr>
<td>Ubiquinone</td>
<td>2.9</td>
<td>5.4</td>
</tr>
<tr>
<td>NAD</td>
<td>7.0</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* Concentrations are expressed as micromoles per gram of protein.
* Data for S. carlsbergensis were taken from Ohnishi et al. (23).

(2) or ill-defined particulate fractions (11, 20). The report by Boulter and Derbyshire (2) on the cytochrome spectra of a large number of fungi, including A. niger, showed a resemblance between the recorded spectra and that of a reduced yeast suspension.

The results presented in this paper confirm these earlier observations; in addition, this report extends the studies to include the properties of a mitochondrial fraction, the electron transfer chain of which is similar to that in mitochondria from yeast and mammalian systems, as concluded in the previous communication (27).

Absorption maxima of the difference spectra revealed bands which may be assigned to cytochromes a + a1, c + c1, and b. The spectra resembled that reported for yeast mitochondria (21, 22) and differed only quantitatively from the cytochrome content of rat liver mitochondria (6). There were indications of a partial loss of cytochrome c from the mitochondria during the isolation procedure, as shown by the fact that added cytochrome c stimulated the oxidation of succinate, citrate, or 2-ketoglutarate by 0 to 20%. With exogenous NADH as substrate, cytochrome c greatly increased oxygen uptake. An analogous effect is observed with intact rat liver mitochondria made permeable to NADH (10, 16, 18).

More recent experiments have shown that the degree of stimulation of oxygen uptake by exogenous cytochrome c in mitochondrial preparations from A. niger is dependent on the pH of the reaction medium and also on the concentration of the added cytochrome c (K. Watson and J. E. Smith, in preparation). Although under the present conditions of assay loss of cytochrome c from the extracted mitochondria is implied, based on the assumption that respiratory control is a measure of the integrity of isolated mitochondria, the high respiratory control ratios observed in A. niger mitochondria would indicate a degree of intactness comparable to that reported for isolated yeast mitochondria (4, 22) and superior to that reported for N. crassa mitochondria (7).

Relatively large amounts of ubiquinone (2 to 3.6 μmoles/g of protein) and NAD (6.2 to 7.2 μmoles/g of protein) were found in the isolated mitochondrial fractions. The only other report of the presence of NAD in fungal mitochondria has been by Ohnishi et al. (23) on the yeast Saccharomyces carlsbergensis.

The role of ubiquinone in mammalian mitochondria has been the subject of intense investigation in recent years (19). Ubiquinone has been identified in whole cells of fungi (5, 15, 17, 24), but studies on the occurrence of ubiquinone in fungal mitochondria have been limited. Anderson (1) reported the restoration of succinate and NADH cytochrome c reductase activity of acetone-extracted respiratory particles of Claviceps purpurea by the addition of ubiquinone 10, and a recent report by Ohnishi et al. (23) has shown that ubiquinone is an obligatory member of the electron transport chain of yeast mitochondria.

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