Mitochondrial Adenosine Triphosphatase of Wild-Type and poky Neurospora crassa

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We have compared the adenosine triphosphatase (ATPase) activity of mitochondria prepared from wild-type Neurospora crassa and from poky, a maternally inherited mutant known to possess defective mitochondrial ribosomes and reduced amounts of cytochromes aa3 and b. poky contains two distinct forms of mitochondrial ATPase. The first is normal in its $K_m$ for ATP, specificity for nucleotides and divalent cations, pH optimum, cold stability, and sensitivity to inhibitors (oligomycin, $N,N$-dicyclohexyl carbodiimide, and adenylyl imidophosphate). The fact that membrane-bound, cold-stable, oligomycin-sensitive ATPase activity is present in poky (with an activity of $1.93 \pm 0.03 \mu$mol/min $\cdot$ mg of protein compared with $1.33 \pm 0.07 \mu$mol/min $\cdot$ mg of protein in the wild-type strain) and also in chloramphenicol-grown wild-type cells suggests that products of mitochondrial protein synthesis play only a limited role in the attachment of the mitochondrial ATPase to the membrane in Neurospora. poky also contains a second form of mitochondrial ATPase, which has an activity of $1.5 \pm 0.2 \mu$mol/min $\cdot$ mg of protein, is oligomycin sensitive but cold labile, and presumably is attached less firmly to the mitochondrial membrane. The two forms, added together, represent a substantial overproduction of mitochondrial ATPase by poky.

In an attempt to understand the way in which genes specify mitochondrial proteins, much attention has been focused upon the poky mutant of Neurospora crassa. poky, first described in 1952 by Mitchell and Mitchell (17), is a maternally inherited mutant with a grossly defective cytochrome chain. It contains very little cytochrome aa3 or b, and, as a result, both cyanide-sensitive respiration and oxidative phosphorylation are reduced by comparison with wild-type Neurospora (11-14, 43). Instead, poky makes use of a derepressed alternate oxidase that branches from the usual electron transport chain at the level of flavoproteins and—although not coupled directly to any phosphorylation site—permits ATP synthesis by substrate-level phosphorylation and probably by oxidative phosphorylation at site I (33).

There is now good evidence that the primary lesion in poky is in the small subunit of mitochondrial ribosomes (9, 10, 26). The resulting deficiency of intact mitochondrial ribosomes leads to a slowing down of mitochondrial protein synthesis and can readily account for the observed abnormalities in cytochrome content, because cytochromes aa3 and b are known to possess mitochondrial-synthesized polypeptides (31, 41, 42, 44).

The effect of the poky mutation upon another major mitochondrial enzyme, mitochondrial adenosine triphosphatase (ATPase), has not yet been examined in detail. Experiments with yeast have established that mitochondrial ATPase, like cytochromes aa3 and b, contains multiple polypeptide chains, some of which (the constituents of the F1 portion and stalk) are synthesized in the cytoplasm whereas others (hydrophobic polypeptides tightly associated with the inner membrane) are synthesized in the mitochondria (32, 36, 38, 39). As a consequence, interfering with mitochondrial protein synthesis in yeast (for example, by adding chloramphenicol) results in inhibition of the assembly of the complete oligomycin-sensitive mitochondrial ATPase complex; instead, oligomycin-resistant, cold-labile F1 ATPase activity accumulates in the cytoplasm (35). Similarly, when the synthesis of the mitochondrial subunits is prevented by mutation, the ATPase becomes oligomycin resistant and can be easily detached from the inner membrane by sonic disruption (8, 22, 28).

The extent to which the biogenesis of mitochondrial ATPase in Neurospora (an obligate aerobe) resembles that in yeast (a facultative anaerobe) is not yet clear. Recently, an inactive
ATPase complex has been isolated from detergent-solubilized submitochondrial particles of *Neurospora* by immunoprecipitation with antibody to purified *Neurospora* F1 ATPase (6). This complex contains approximately 14 polypeptides, and differential labeling in the presence of chloramphenicol and cycloheximide indicates that at least 2 of the polypeptides are synthesized mitochondrialy. However, one specific ATPase component known to be synthesized mitochondrialy in yeast—the membrane proteolipid that binds $N,N$-dicyclohexyl carbodiimide (DCCD) (32)—appears to be synthesized in the cytoplasm in *Neurospora* (30).

The experiments reported in the present paper were undertaken to characterize the mitochondrialy ATPase of *Neurospora* in its active, membrane-bound form and to see to what extent the *poky* mutation leads to defects in the ATPase. A preliminary account of this work has already been published (S. E. Mainzer and C. W. Slayman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K15, p. 139).

**MATERIALS AND METHODS**

**Growth of Neurospora.** Wild-type strain RL21a and *poky* strain NSX/ were used in these experiments; *f* is a nuclear-gene suppressor of the *poky* mutation that increases the growth rate without restoring the wild-type respiratory system (18). Conidia were inoculated at a density of $10^6$ per ml into Vogel minimal medium (40) containing 2% sucrose as the carbon source and were grown with vigorous aeration at 25°C for 15 h (wild type) or 21 h (*poky*).

**Preparation of mitochondria and submitochondrial particles.** Most experiments were carried out with mitochondria isolated by the method of Lambowitz et al. (12). Where specified in the text, the mitochondria were further purified by sucrose gradient centrifugation (16). For the preparation of submitochondrial particles, mitochondria (60 mg of protein) were suspended in 2.0 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)-SO4 (Sigma), pH 7.45. The suspension was disrupted by sonic treatment for 4 min with a Heat System W200 R cell disrupter equipped with a special microtip (power setting, 5; 40% duty cycle giving 1.6 min of total sonic treatment time), and submitochondrial particles were collected by the method of Tzagoloff (34).

**Enzyme assays.** ATPase activity was assayed by three alternative procedures, as follows. (i) The liberation of inorganic phosphate was determined in 1.0 ml of reaction mixture containing 5 mM $N_A$ATP, 5 mM MgCl2, 50 mM Tris-glycylglycine buffer (pH 8.25), and an ATP regenerating system (5 mM phospho-oligopyruvate [PEP]–50 µg of pyruvate kinase [Sigma type II kinase]). The reaction was started by the addition of 15 to 100 µg of mitochondrial protein. After 5 or 10 min, the assay was terminated by the addition of 200 µl of 55% trichloroacetic acid, and the tubes were centrifuged at 2,000 × *g* for 5 min. A portion of the supernatant fraction was then assayed for inorganic phosphate by the method of Dryer et al. (4).

(ii) Alternatively, ATPase activity was measured spectrophotometrically in a coupled enzyme assay system. In this case, the reaction mixture contained 5 mM $N_A$ATP, 5 mM MgCl2, 50 mM Tris-glycylglycine buffer (pH 8.25), ATP regenerating system (as above), 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), and 50 µg of lactic dehydrogenase, in a total volume of 3.0 ml. The reaction was started by the addition of mitochondria, and the decrease in absorbance at 340 nm (due to the oxidation of NADH) was monitored with a Zeiss PMQ II spectrophotometer equipped with a Kipp and Zonen BD11 lin-log recorder.

(iii) Finally, ATPase activity was measured by the decrease in pH that accompanies the hydrolysis of ATP. The reaction mixture consisted of 0.1 mM $N_A$ATP, 0.1 mM MgCl2, and 1.0 mM tricine buffer (pH 8.25), in a 2.3-ml water-jacketed vessel. The reaction was started by the addition of mitochondria (1 to 3 µl of a suspension containing 60 mg of protein per ml), and the rate of change of pH was measured with a Radiometer 25 pH meter attached to a Kipp and Zonen recorder. The total change in pH did not exceed 0.01 pH unit.

All ATPase assays were carried out at 30°C unless otherwise specified. Phosphatase activity was determined with 5 mM *p*-nitrophenyl phosphate as substrate in the presence of 5 mM MgCl2 and 50 mM Tris-glycylglycine buffer, pH 6.9 to 9.25. In some experiments, the liberation of inorganic phosphate was assayed by the method of Dryer et al. (4); in others, the appearance of *p*-nitrophenol was monitored spectrophotometrically, by adjusting the solution to an alkaline pH and measuring the absorbance at 410 nm.

**Protein assay.** Protein was measured by the method of Lowry et al. (15) with bovine serum albumin as the standard.

**Reagents.** Nucleoside triphosphates, *p*-nitrophenyl phosphate, PEP, NADH, pyruvate kinase, lactic dehydrogenase, tricine, glycylglycine, oligomycin, DCCD, adenylyl imidodiphosphate (AMP-PNP), carbonyl cyanide *m*-chlorophenylhydrazone, and chloramphenicol were obtained from Sigma Chemical Co.

**RESULTS**

**Effect of pH on ATPase activity.** Figure 1A shows the ATPase activity of *Neurospora* mitochondria over the pH range 6.9 to 9.5. In both wild type and *poky*, the major peak of activity occurred at pH 8.25, with a smaller and more variable peak between pH 7.25 and 7.50. In the middle of the pH range (from pH 7.25 to 8.75), the ATPase activity of both strains was nearly completely inhibited by oligomycin (25 µg/mg of protein [Fig. 1B]), as expected for mitochondrial ATPase. In the same range, phosphatase activity was extremely low (0.02 to 0.06 µmol/min/mg of protein, assayed with *p*-nitrophenyl phosphate as substrate). Above pH 8.75, oligomycin-resistant ATPase (Fig. 1B) and phosphatase increased sharply; both of these activities could be reduced by passage of the mitochondria through a continuous sucrose gra-
dient, but because all further work was carried out at pH 8.25 (the optimum for the mitochondrial ATPase), conditions under which contamination by oligomycin-resistant ATPase and by phosphatase was less than 10%, the sucrose gradient step was normally omitted. Addition of 3 μM carbonyl cyanide m-chlorophenyl hydrzone did not stimulate ATPase activity at pH 8.25, presumably because the mitochondria were already fully uncoupled.

One unexpected aspect of the results shown in Fig. 1 was the substantial amount of oligomycin-sensitive ATPase activity present in poky mitochondria. In this particular experiment, the specific activity of poky was 77% greater than that of wild type (2.1 and 1.3 μmol/min · mg of protein, respectively). In other experiments, the specific activity averaged 1.93 ± 0.03 μmol/min · mg of protein in poky (mean ± standard error of the mean for 10 determinations) and 1.33 ± 0.07 μmol/min · mg of protein in wild type (for 13 determinations).

For convenience, the strain of poky used for these experiments carried the nuclear suppressor f, which increases the growth rate without restoring the wild-type cytochrome composition (18). To check the possibility that the f suppressor mutation might be responsible for the surprising amount of oligomycin-sensitive ATPase activity seen in poky, mitochondria were isolated from 21-h cells of an f-minus strain of poky and proved to have essentially the same characteristic: an ATPase activity at pH 8.25 of 2.84 μmol/min · mg of protein (even higher than that usually observed in f-plus poky), which was 85% sensitive to oligomycin (25 μg/mg of protein). All subsequent experiments were carried out with poky containing f.

**Effect of inhibitors of ATPase activity.**

Because sensitivity to oligomycin can provide information about the association of mitochondrial ATPase with the inner membrane, it was important to compare the effects of a range of oligomycin concentrations on wild-type and poky mitochondria. The results of an experiment of this kind are shown in Fig. 2A. The ATPase activity in poky proved, if anything, to have a slightly increased sensitivity to oligomycin: half-maximal inhibition occurred at 0.5 μg/mg of protein in poky compared with 1.0 μg/mg of protein in the wild type. Overall, 92 to 95% of the total ATPase activity was inhibited in both strains by the highest oligomycin concentration in this experiment (50 μg/mg of protein); in other experiments, the remaining 5 to 8% of activity was not abolished by concentrations as high as 200 μg/mg of protein.

Two other mitochondrial ATPase inhibitors were tested (Fig. 2B): DCCD, which, like oligomycin, inhibits only the membrane-bound form of the enzyme (25), and AMP-PNP, which competes with ATP and inhibits both the soluble and membrane-bound forms (21). In both cases,
half-maximal inhibition occurred at the same concentration in the wild type and poky (8 μg/mg of protein for DCCD and 6 μM for AMP-PNP).

Measurement of $K_m$. ATPase activity was determined for both wild-type and poky mitochondria over a range of ATP concentrations, from 0.08 to 7.5 mM. Figure 3 is an Eadie-Hofstee plot of the data from one such experiment. The two preparations had very similar $K_m$ values (0.33 mM for the wild type and 0.29 mM for poky), but, as expected from results reported above, different maximal activities (1.2 μmol/min · mg of protein for the wild type and 2.0 μmol/min · mg of protein for poky). In this particular experiment, ATPase activity was assayed as the liberation of inorganic phosphate, but essentially the same $K_m$ values (between 0.1 and 0.3 mM) were obtained by the pH or spectrophotometric assays.

Substrate specificity. The ability of other nucleoside triphosphates to substitute for ATP is shown in Table 1. Generally, the wild-type and poky enzymes were very similar in both cases; ATP was the best substrate; GTP and ITP were hydrolyzed almost as rapidly, and UTP, CTP, and TTP were poor substrates.

Effect of salts and of divalent cations. The effect of increasing salt concentrations in the presence and absence of Mg$^{2+}$ is shown in Fig. 4 for both wild-type and poky ATPases. To minimize the starting salt concentration in this experiment, the reaction was carried out in 1 mM tricine buffer (pH 8.25) at an ATP concentration of 0.1 mM, and ATP hydrolysis was measured by the pH assay. In the absence of Mg$^{2+}$, ATPase activity was very low and was not increased upon addition of KCl or NaCl (up to 75 mM). In the presence of Mg$^{2+}$, base-line activity was higher, and the addition of KCl or NaCl produced a further stimulation of three- to fivefold, with a half-maximal effect at approximately 8 mM salt both in the wild type and poky. Simultaneous addition of KCl and NaCl gave no evidence of synergism. When the buffer concentration was increased to 50 mM, the salt effect was no longer seen.

The Mg$^{2+}$ requirement of ATPase, which is shown in Fig. 4, was explored further by testing the ability of other divalent cations to substitute for Mg$^{2+}$ (Table 2). In both wild-type and poky mitochondria, the same order of effectiveness was observed, with Mn$^{2+}$ producing an even greater stimulation than Mg$^{2+}$, Co$^{2+}$ giving some

<table>
<thead>
<tr>
<th>Table 1. Substrate specificity$^a$</th>
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<tr>
<td>Nucleoside triphosphate</td>
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</tr>
<tr>
<td>ATP</td>
</tr>
<tr>
<td>GTP</td>
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<tr>
<td>ITP</td>
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<td>UTP</td>
</tr>
<tr>
<td>CTP</td>
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<td>TTP</td>
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$^a$ Activity was assayed by the liberation of P$_i$ in a reaction mixture consisting of 50 mM Tris-glycylglycine (pH 8.25) and 0.1 to 10 mM magnesium-nucleoside triphosphate. For each nucleoside triphosphate and each strain, the maximal activity was calculated by a computer fit of the data to the Michaelis-Menten equation. Activities are reported as the percentage of activity with ATP as substrate. The control rates with ATP were 1.0 μmol/min · mg of protein for the wild type and 1.7 μmol/min · mg of protein for poky.
Table 2. Effects of divalent cations on the ATPase activity of wild-type and poky mitochondria

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>Wild type</th>
<th>poky</th>
</tr>
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<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>144</td>
<td>220</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>56</td>
<td>84</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

*ATPase activity was assayed by measuring the release of H$^+$ ions that accompanies the hydrolysis of ATP, as described in the text. The reaction mixture consisted of 0.1 mM divalent cation, 0.1 mM ATP, and 1.0 mM tricine buffer (pH 8.25); other experiments (data not shown) confirmed that maximal activity was obtained at a 1:1 ratio of divalent cation to ATP. Activities are reported as the percentage of activity with Mg$^{2+}$. The control rates with Mg$^{2+}$ were 0.57 μmol/min·mg of protein for the wild type and 0.86 μmol/min·mg of protein for poky (the low rates reflecting the fact that the assay was carried out at 0.1 mM ATP).

activity, and Ca$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$ serving as very poor substitutes.

**Measurement of activation energy.** A very sensitive way to detect abnormalities in a membrane-bound enzyme complex is to analyze enzyme activity as a function of temperature. We therefore determined the ATPase activities of wild-type and poky mitochondria over a range of temperatures, from 30 to 60°C. When freshly isolated mitochondria were compared, three small but significant differences were observed, as follows. (i) Total ATPase activity and oligomycin-sensitive ATPase activity of poky mitochondria were higher than that of wild-type mitochondria at all temperatures tested, although oligomycin-insensitive activity was the same for the two strains. (ii) Maximal ATPase activity was observed at 55°C in the wild type and at 60°C (or above) in poky. (iii) Arrhenius plots revealed a 30% lower activation energy for the poky ATPase (33.7 kJ/mol) than for the wild-type ATPase (47.2 kJ/mol) (Fig. 5). These differences persisted in mitochondria that had been frozen in acetone-Dry Ice and thawed 3 h later, but diminished when the mitochondria were stored at −70°C for one week; in the latter case, maximal activity in both strains was observed at 55°C, and the activation energies were 42.1 kJ/mol for poky and 37.9 kJ/mol for wild type.

**Retention of ATPase activity in submitochondrial particles.** The results reported up to this point indicate that poky, in spite of its known defect in mitochondrial protein synthesis, possesses an elevated specific activity of mitochondrial ATPase with only minor differences in kinetic properties and in activation energy. Furthermore, the fact that the poky ATPase is oligomycin sensitive implies that it is bound in a relatively normal way to the mitochondrial membrane. A second way to investigate binding is by the preparation of submitochondrial particles. In yeast, for example, disruption by sonic treatment leaves the wild-type mitochondrial ATPase in the particulate fraction but releases the ATPase of petite mutants into the supernatant fraction (28). To examine this point in *Neurospora*, mitochondria were prepared from the wild-type strain and from poky, disrupted by sonic treatment for 1.6 min, and centrifuged, and the various fractions were assayed for ATPase activity (Table 3). In both strains, oligomycin-sensitive ATPase activity was recovered in good yield in the particulate fraction (128% in the wild type and 99% in poky), indicating firm binding of the ATPase to the mitochondrial membrane.

**Response of ATPase activity to incubation in the cold.** One well-established property of mitochondrial ATPase preparations from other organisms is their complex response to cold incubation. The complete oligomycin-sensitive ATPase complex, including the membrane subunits, is cold stable, but solubilized F1 ATPase (which is insensitive to oligomycin) loses its activity rapidly when incubated at 0°C (24).

![Fig. 5. Arrhenius plot of the ATPase activity of wild-type (x) and poky (C) mitochondria. Activity was measured in the presence and absence of oligomycin (25 μg/mg of protein) at pH 8.25 and at temperatures of 30 to 50°C. Linear (increasing) portions were fitted by the method of least squares; correlation coefficients were greater than 0.9.](http://jb.asm.org/)
Cold stability can therefore be used as a third criterion of normal attachment of F1 to the mitochondrial membrane. All of the experiments reported above for Neurospora mitochondrial ATPase were carried out with mitochondria that had been prepared at 0 to 4°C, conditions that would completely inactivate F1 ATPase. It is clear, then, that the elevated mitochondrial ATPase activities seen in poky are normal in their cold stability. We thought it useful, however, to see whether additional cold-labile ATPase activity might exist in wild-type and especially in poky mitochondria. For this purpose, mitochondria from both strains were prepared at room temperature, divided into two portions, and kept for 4 h at 25 and 0°C, respectively, with periodic assays of ATPase activity (Fig. 6).

In the wild-type mitochondria prepared at room temperature, total ATPase activity was somewhat higher than usual (1.9 umol/min · mg of protein, compared with 1.33 ± 0.07 umol/min · mg of protein for cold-prepared mitochondria); it was more than 90% inhibited by oligomycin, and both the major oligomycin-sensitive fraction and the minor oligomycin-insensitive fraction remained constant for the entire 4-h period of the experiment, regardless of whether the mitochondria were kept at room temperature (Fig. 6, upper left) or at 0°C (Fig. 6, lower left). Warm-prepared poky mitochondria also exhibited an unusually high ATPase activity (4.0 umol/min · mg of protein, compared with values of 1.93 ± 0.03 umol/min · mg of protein seen with cold-prepared poky mitochondria). The response during the 4-h incubation period was quite different in poky, however. When poky mitochondria were incubated at room temperature, total ATPase activity remained relatively constant, but there was a steady increase in the fraction insensitive to oligomycin (Fig. 6, upper right). It was equally striking that when poky mitochondria were transferred to 0°C, there was a pronounced loss of total ATPase activity over the 4-h incubation period until, by the end of the incubation, activity had declined almost to the range characteristic of cold-prepared poky mitochondria. It appears, therefore, that in addition to the large amount of relatively normal mitochondrial ATPase activity in poky, there is a further portion that is decided abnormal; it is cold labile and at room temperature gradually becomes insensitive to oligomycin during prolonged incubation.

**Effect of growth in the presence of chloramphenicol.** As an independent check on the role of mitochondrial protein synthesis in the production of the Neurospora mitochondrial ATPase, cells were grown in the presence of chloramphenicol. Of course, the cold-sensitive ATPase activity of wild-type Neurospora mitochondria would be expected to decrease when growth in the presence of chloramphenicol, and this is what happens (Fig. 7). This indicates that the cold-sensitive activity is labile and is therefore sensitive to chloramphenicol. The chloramphenicol-insensitive total activity of warm-prepared wild-type mitochondria is about 2.0 umol/min · mg of protein, which is comparable to the cold-sensitive activities of cold-prepared mitochondria. The chloramphenicol-insensitive activity of cold-prepared poky mitochondria is about 4.0 umol/min · mg of protein, which is about twice as high as the cold-sensitive activities of cold-prepared wild-type mitochondria. The chloramphenicol-insensitive activity of warm-prepared poky mitochondria is not significantly different from the cold-sensitive activity of cold-prepared wild-type mitochondria.

**Table 3. ATPase activity in sub mitochondrial particles**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units (umol/min)</th>
<th>Sp act (umol/min · mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Plus oligomycin</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>120</td>
<td>1.19</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>145</td>
<td>2.38</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>17</td>
<td>0.49</td>
</tr>
<tr>
<td>poky</td>
<td></td>
<td></td>
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<tr>
<td>Mitochondria</td>
<td>86.5</td>
<td>2.22</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>85.4</td>
<td>3.06</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>0.9</td>
<td>0.06</td>
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</table>

a Mitochondria (101 mg in the case of the wild type and 39 mg in the case of poky) were suspended in 10 mM Tris-sulfate (pH 7.45) and disrupted by sonic treatment as described in the text. Between 36 and 46% of the total mitochondrial protein was usually released by sonic treatment. The suspension was centrifuged at 110,000 × g for 60 min. The resulting particulate and supernatant fractions were assayed for ATPase activity at pH 8.25 in the presence of 5 mM MgCl2 and 5 mM ATP, and in the presence and absence of oligomycin (13 to 40 μg/mg of protein), as described in the legend to Fig. 1. Protein recovery was 101% for poky and 95% for the wild type.

**Fig. 6. ATPase activity of wild-type (x) and poky (○) mitochondria prepared at room temperature and kept for 4 h either at room temperature (warm → warm) or at 0°C (warm → cold). Activity was assayed at pH 8.25 as described in the legend to Fig. 1, in the presence and absence of oligomycin (25 μg/mg of protein).**
chloramphenicol (4 mg/ml). Previous experiments have shown that such cells, like poky, contain greatly reduced amounts of cytochromes aa3 and b (14). Table 4 is a summary of the ATPase activities of cold-prepared mitochondria from chloramphenicol-grown wild type, poky, and (for comparison) chloramphenicol-grown yeast. In both strains of Neurospora, growth in the presence of chloramphenicol did not significantly alter the amount of cold-stable ATPase activity (Table 4). Furthermore, the ATPase remained oligomycin sensitive, although high concentrations of oligomycin (100 μg/ml of protein) were required to give complete inhibition. (The data in Table 4 show partial inhibition with 30 to 59 μg of oligomycin per mg of protein.) By comparison, mitochondria isolated from chloramphenicol-grown yeast contained greatly reduced amounts of cold-stable ATPase that was only 30% inhibited by oligomycin. Thus, there appears to be a significant difference between Neurospora and yeast with respect to the role of mitochondrial protein synthesis.

DISCUSSION

Properties of the Neurospora mitochondrial ATPase. The results reported in this pa-

per demonstrate that Neurospora mitochondria contain substantial membrane-bound ATPase activity, as would be expected from the fact that Neurospora is an obligately aerobic fungus that produces most of its ATP by oxidative phosphorylation (33). Generally, the Neurospora enzyme is similar to the ATPase isolated from yeast and mammalian mitochondria. All are sensitive to the inhibitors oligomycin, DCCD, and AMP-PNP, and all have Kₘ values for Mg-ATP in the range 0.1 to 0.3 mM (1–3, 20, 27, 29, 37). There are some minor kinetic differences. The Neurospora ATPase has a pH optimum between 8.0 and 8.5, like beef heart (37) and rat liver (3) but unlike yeast (2, 27), for which maximal activity is seen above pH 9.0. However, in its nucleotide specificity and divalent cation requirements, the Neurospora ATPase is closer to yeast than to the mammalian enzymes. Yeast and Neurospora hydrolyze ATP, GTP, and ITP at nearly identical rates, whereas rat liver and beef heart show a pronounced specificity for ATP (3, 37). Also, the yeast and Neurospora enzymes prefer Mn²⁺ to Mg²⁺ (2); mammalian mitochondrial ATPases prefer Mg²⁺, although they do function in the presence of Mn²⁺, Co²⁺, or Ca²⁺ (5).

Comparison of wild type and poky. The most significant outcome of this study is the finding that the mitochondrial ATPase of poky, when characterized in the membrane-bound state in cold-prepared mitochondria, is nearly indistinguishable from that of the wild type. Thus, in spite of a clear-cut defect in mitochondrial protein synthesis that grossly impairs the production of cytochromes aa₃ and b (12, 31, 41, 42, 44, 45), poky makes a substantial amount of mitochondrial ATPase that is cold stable, is sensitive to oligomycin, and has normal kinetic properties.

Whether the poky ATPase functions normally in oxidative phosphorylation is not yet clear. Cytochrome-linked phosphorylation in isolated mitochondria is only about one-half as efficient in poky as in the wild type. For example, in the study of Lambowitz et al. (14), wild-type mitochondria respiring on succinate gave ratios of phosphorus to oxygen of 1.3, whereas poky mitochondria respiring on succinate in the presence of salicyl hydroxamic acid (to block the alternate oxidase) gave ratios of phosphorus to oxygen of about 0.75. This difference could reflect an abnormality in the functioning of the ATPase complex itself, but it could arise equally well from lesions elsewhere in the inner membrane. (For example, isolated poky mitochondria have been shown to be more permeable to small ions than are isolated wild-type mitochondria [7], and according to one leading theory of oxidative phosphorylation [19], increased "leakiness" of the

<table>
<thead>
<tr>
<th>Table 4. Mitochondrial ATPase activity in chloramphenicol-grown cells*</th>
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<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td><strong>Wild-type Neurospora</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Chloramphenicol grown</td>
</tr>
<tr>
<td><strong>poky Neurospora</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
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<tr>
<td><strong>Yeast</strong></td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>Chloramphenicol grown</td>
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* Mitochondria were isolated as described in the text from wild-type and poky Neurospora grown in the presence of chloramphenicol (4 mg/ml). In addition, mitochondria were isolated by the method of Duell et al. (5) from yeast (Saccharomyces cerevisiae) grown for 17 h in 0.8% glucose–1% yeast extract–1% peptone–40 mg of adenine per liter–4 mg of chloramphenicol per ml. ATPase activity was assayed at pH 8.25 as described in the legend to Fig. 1 in the presence of 5 mM ATP and 5 mM MgCl₂, and in the presence and absence of oligomycin (45 μg/mg of protein for wild-type Neurospora, 31 μg/mg of protein for poky, and 59 μg/mg of protein for yeast).
inner membrane should dissipate the potential generated by the redox chain, thereby decreasing the efficiency of phosphorylation.

The implications of these findings for the biogenesis of the mitochondrial ATPase complex remain to be worked out. The relative normality of the poky ATPase in cold-prepared mitochondria and the fact that very similar results were obtained with cold-prepared mitochondria from chloramphenicol-grown cells indicate that products of mitochondrial protein synthesis play only a limited role in the functioning of the Neurospora mitochondrial ATPase, in contrast with their clear-cut role in attaching F1 to the membrane and conferring oligomycin sensitivity in yeast (38). In pursuing this question, it will be important to purify an active membrane-bound ATPase from Neurospora, as well as an active F1 ATPase, and to compare the subunits present in these complexes in wild type, poky, and chloramphenicol-grown wild type. In this way, it should be possible to clarify the role of the mitochondrially synthesized polypeptides described by Jackl and Sebald (6) and to understand the basis for oligomycin sensitivity.

A second significant result of this study is the finding that, in addition to its cold-stable, oligomycin-sensitive ATPase, poky contains a significant amount of ATPase that is associated with the mitochondria in an oligomycin-sensitive manner but is cold labile. Taken together, the total specific activity of the two forms of ATPase in poky (4.0 μmol/min·mg of protein in warm-prepared mitochondria) exceeds the specific activity of the ATPase of wild-type mitochondria (1.9 μmol/min·mg of protein), suggesting that mitochondrial ATPase may belong to the group of mitochondrial proteins (including cytochrome c and the alternate terminal oxidase [13]) that are produced in elevated amounts in poky. Two alternative explanations of the specific activity data can be ruled out on the basis of existing evidence, as follows. (i) If poky mitochondria were abnormally fragile, damage during the isolation procedure might lead to loss of soluble matrix protein and consequently to enrichment of ATPase attached to the inner membrane. This explanation is unlikely, however, because mitochondria from the wild type and from poky display identical osmotic behavior in sucrose solutions and have the same sucrose-impermeable space (7). (ii) If poky cells contained a normal number of ATPase complexes inserted into a decreased number of mitochondria, the result would be an increase in the specific activity of the ATPase. This explanation, too, seems unlikely, because the standard preparation procedure (treatment of cells with snake enzyme to weaken their cell walls, followed by gentle homogenization) routinely releases 1.5- to 2-fold more mitochondrial protein from poky than from the wild-type strain (S. E. Mainzer and K. E. Allen, unpublished data). The possibility remains that the increased specific activity of the poky ATPase reflects an increased turnover number resulting, for example, from abnormal binding of an inhibitory subunit or from abnormal association of the ATPase with the mitochondrial membrane. Whether this is the case or whether ATPase complexes are truly produced in elevated amounts in poky should become evident from purification of the ATPase and from studies of its subunit composition.

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