MITOCHONDRIAL PROTEIN SYNTHESIS AND CYANIDE-RESISTANT RESPIRATION IN COPPER-DEPLETED, CYTOCHROME OXIDASE-DEFICIENT *NEUROSPORA CRASSA*

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1. Introduction

In mitochondria from the maternally inherited *mi-1* and *mi-3* mutants of *Neurospora crassa* and from wild-type cells grown in the presence of chloramphenicol, the content of cytochrome aa_3 is strongly reduced, and an additional cyanide-insensitive, hydroxamate-sensitive oxidase is present giving rise to a branched respiratory chain [1–10]. In the present report, it will be shown that a similar branching of the respiratory chain occurs in wild-type *Neurospora* cells grown in a copper-deficient medium. In contrast to mutant and chloramphenicol-treated cells, copper-depleted cells show normal mitochondrial protein synthesis.

2. Materials and methods

A 20-fold concentrate of the final copper-deficient Vogel's minimal medium [11] was obtained by means of a chelating cation exchanger (Chelex 100, 200–400 mesh, BioRad Laboratories, Richmond, Calif. USA) in the following way: The concentrate with all trace elements omitted was passed at 100 ml/hr through a column (2 X 2 cm) of the exchanger previously transferred to the Ca-form [12]. Then, the trace elements (copper omitted) were added, using analytical grade salts (E. Merck, Darmstadt, Germany). After dilution with distilled water, the medium was sterilized. Alternatively, the extraction procedure of Giorgio et al. [13] was applied, leading to similar results. In this medium, *N. crassa* wild type was cultured as described earlier [14], except that the inoculum was reduced to 5×10^4 conidia per ml, in order to reduce the amount of copper introduced by the conidia.

Distilled water with a conductance of 10^6 ohm^{-1} cm^{-1} was obtained from deionized water by distilling in an all-quartz apparatus. All glassware in contact with copper-deficient solutions was cleaned as described by Giorgio et al. [13]; a 5% detergent solution (RBS 25, Carl Roth OHG, Karlsruhe, Germany) at 70°C was used instead of dichromate–sulfuric acid.

For protein labelling, [U-^{14}C]L-leucine (50 μCi/ℓ of culture medium, 330 mCi/mmol) was added directly to the growing culture 24 hr after inoculation. After further 200 min of growth, cycloheximide (Carl Roth OHG, Karlsruhe, Germany) was added (0.1 g/ℓ), followed after 2 min by 1 mCi/ℓ [4,5-^{3}H]L-leucine (36 Ci/mmol, The Radiochemical Centre, Amersham, England) [14]. Cycloheximide was previously freed from traces of copper by passing a 1% solution with 200 mM NaCl through a column (1 X 2 cm) of Chelex 100 in the sodium form.

Preparation of mitochondria and of mitochondrial membranes, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and measurements of radioactivity and of oxygen consumption were performed as described previously [14,15]. Counting efficiencies were 45% for ^{14}C and 20% for ^{3}H. The difference spectra of whole mitochondria were obtained with a special split-beam spectrophotometer designed by M. Klingenberg. The cytochrome contents were calculated by the procedure described by von Jagow et al. [7].

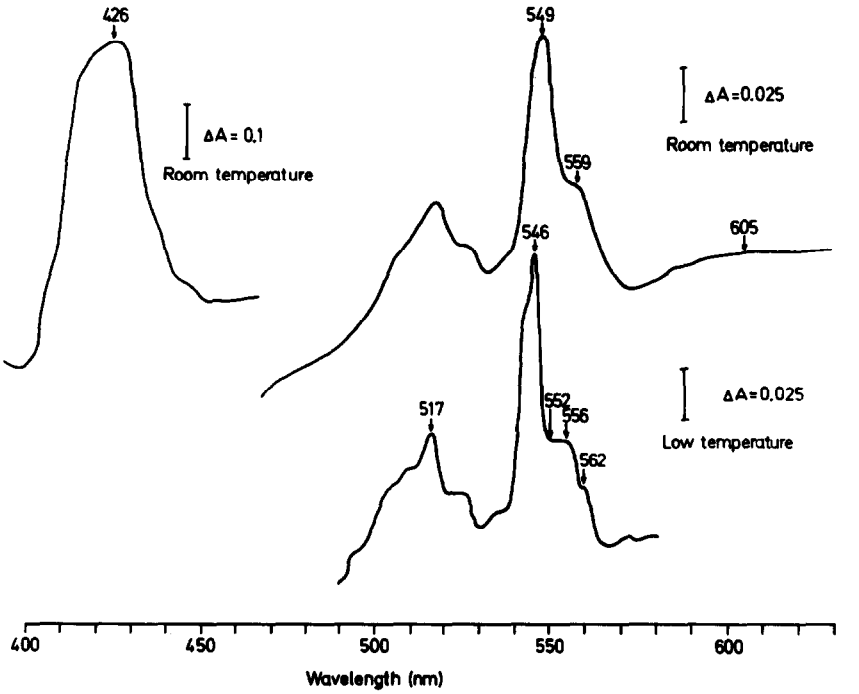

Fig. 1. Difference spectra (dithionite reduced *vs.* air oxidized) of mitochondria (5.4 mg protein/ml) from copper-depleted *Neurospora* cells at room temp. (path length 0.5 cm) and at the temperature of liquid nitrogen (path length 0.1 cm).

Table 1
Respiratory activity and inhibitor sensitivity of mitochondria from copper-depleted *Neurospora* cells with 1 mM external NADH as a substrate.

Additions	Oxygen uptake (μatoms O × min^{-1} × g protein^{-1})	% of control
None	670 ± 60	100
Salicylhydroxamate (2 mM)	140 ± 20	20
Salicylhydroxamate (2 mM) + FCCP (1 μM)	200 ± 30	30
KCN (1 mM)	630 ± 70	94
Antimycin A (2 μM)	660 ± 40	98
Salicylhydroxamate (2 mM) + KCN (1 mM)	40 ± 30	6
Salicylhydroxamate (2 mM) + antimycin A (2 μM)	50 ± 30	8

Protein concentration was 0.5 mg/ml. FCCP = carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The errors given are standard deviations from means of at least four determinations.

3. Results

The cellular concentration after 24 hr of growth in copper-deficient medium was 10 g wet weight per liter of culture. Similar yields were obtained using copper-sufficient medium. Difference spectra (dithionite reduced *vs.* air oxidized) of whole mitochondria from copper-depleted cells are shown in fig. 1. Cytochrome aa_3 (605 nm) is reduced to a level below the detection limit (0.02 μmol/g protein). In contrast, the *b*- and *c*-type cytochrome contents are not substantially altered: cytochrome b_K (556 nm), 0.37 μmol/g protein; cytochrome b_T (562 nm), 0.31 μmol/g protein; cytochrome c_1 (552 nm), 0.37 μmol/g protein; cytochrome *c* (546 nm), 1.42 μmol/g protein. This cytochrome pattern resembles that of the *mi-3* mutant [7].

The rates of oxygen consumption of mitochondria from copper-depleted cells with external NADH as a substrate and its response to inhibitors is shown in table 1. The absolute respiration rate in the absence of inhibitors is comparable to that in normal cells [7, 9, 14]. Respiration is only slightly affected by cyanide

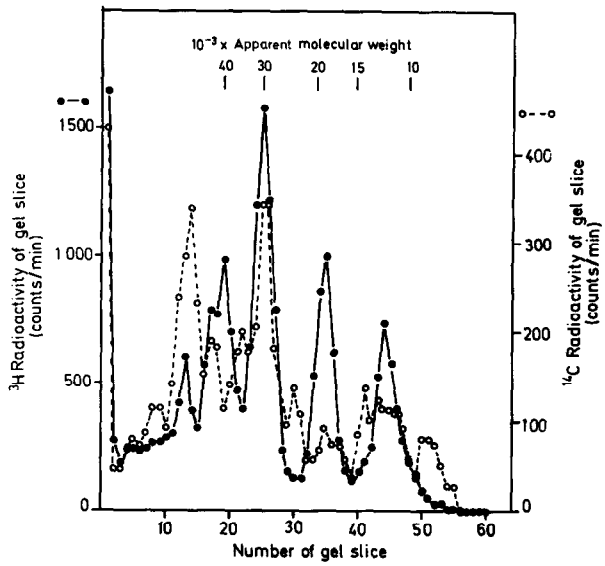

Fig. 2. Distribution of radioactivity after electrophoretic separation of mitochondrial membrane protein from copper-depleted *Neurospora* cells labelled sequentially with [^{14}C]leucine (o---o) in the absence of cycloheximide and with [^{3}H]leucine (•——•) in the presence of cycloheximide as described in the Methods section. The gel was calibrated by electrophoresis in parallel of proteins of known molecular weights [16].

or antimycin A, but fairly inhibited by salicylhydroxamate. The residual activity in the presence of salicylhydroxamate is almost entirely cyanide- and antimycin A-sensitive and shows a slight energy coupling (respiratory control 1.5) as demonstrated by the stimulatory effect of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP).

In the experiments shown in fig. 2, cells were sequentially treated with a pulse of [^{14}C]leucine (uniform labelling of all proteins), with cycloheximide, and with [^{3}H]leucine, as described in the Methods section. Under these conditions the [^{3}H]leucine will label only proteins synthesized on the mitochondrial ribosomes [16–19].

In mitochondrial membranes isolated from copper-depleted cells, specific radioactivities achieved were 98 000 cpm X mg protein^{-1} for ^{14}C and 352 000 cpm X mg protein^{-1} for ^{3}H. The cycloheximide-resistance labelling of the total mitochondrial membrane protein is calculated to be 40% of the labelling without cycloheximide. Similar values have been obtained under similar conditions using copper-sufficient medium [17, 19]. Sodium dodecylsulfate gel electrophoresis of the mitochondrial membrane protein from labelled cells led to the patterns shown. The distribution of the ^{14}C control label is similar to that obtained in copper-sufficient cells [16]. The ^{3}H radioactivity, representing protein of mitochondrial origin, shows four major peaks, which correspond to proteins with molecular weights of approx. 12 000, 20 000, 30 000 and 40 000. This result is similar to that obtained from copper-sufficient cells, in the presence of cycloheximide [15, 16], differing only in the relative peak areas. One may conclude that in copper-depleted cells, mitochondrial protein synthesis is not substantially affected qualitatively and quantitatively.

4. Discussion

Deficiency in cytochrome aa_3 as a consequence of deficiency in copper has been observed in mammals and in yeasts [20–22]. The experiments presented here show that in *N. crassa*, this is accompanied by a branching of the respiratory chain, due to the appearance of an additional cyanide-insensitive, hydroxamate-sensitive oxidase similar to that in the cytochrome-aa_3-deficient cells of the *mi-1* and *mi-3* mutants and of chloramphenicol-treated wild-type [5–10].

In the *mi-1* and *mi-3* mutants, where the mitochondrial genome is altered [23], and in the presence of chloramphenicol, which is known to inhibit mitochondrial protein synthesis in *N. crassa in vivo* [19], the deficiency in cytochrome oxidase is most probably caused by a lack of synthesis of that part of the protein moiety of the enzyme originating from the mitochondrial protein synthesizing system [11, 15, 24, 25]. A deficiency in cytochrome b_T has also been observed in these cases (except in *mi-3*) [6–8], probably as a consequence of the mitochondrial origin of a cytochrome *b* apoprotein as described recently [26].

In this report it is shown that mitochondrial protein synthesis is intact in copper-depleted cells, so most probably the components of cytochrome oxidase with mitochondrial site of synthesis are still present under these conditions. The deficiency in cytochrome aa_3 must have a different cause. The fact that cytochrome oxidase is a copper protein [27] explains

the loss of enzymic activity, but not the disappearance of the cytochrome aa_3 difference spectrum. Experiments with yeasts and with rabbits have shown that the recovery of cytochrome aa_3 after addition of copper is a slow process, inhibited by cycloheximide [28–30]. This suggests that protein synthesis is involved in this recovery process. Thus, copper deficiency might repress the synthesis of either a protein component of cytochrome oxidase of extramitochondrial origin, or of some other protein necessary for the assembly of the enzyme or for the synthesis of heme *a*. An alternative hypothesis would be that copper is introduced at a specific step during the assembly of the enzyme, and that the intermediate (or one of the intermediates) formed in the absence of copper is not stable and is therefore not available after addition of copper.

The hydroxamate-sensitive oxidase is observed in *N. crassa* whenever the content of cytochrome oxidase is reduced. Possibly, induction of this oxidase is an adaptive mechanism, compensating an eventual loss of cytochrome oxidase activity. This is demonstrated by the fact that mitochondrial respiration and cellular growth are not affected in copper-depleted cells. *N. crassa* is obligatorily aerobic, and fermentation is not available for such a compensation.

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