Copper accumulation in the cell-wall-deficient slime variant of *Neurospora crassa*

Comparison with a wild-type strain

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The copper-uptake process in the cell-wall-deficient slime variant of the fungus *Neurospora crassa* was compared with that in a wild-type strain. In both organisms investigated most of the copper is taken up from the culture medium during the exponential growth period. The wild-type strain, however, accumulates much more copper than does the slime variant. The influence of the copper concentration in the culture medium on the amounts of copper accumulated intracellularly suggests separate ways of copper import used by the two morphologically different *N. crassa* strains. Copper analyses of three different cytosolic fractions as a function of growth time or exogenous copper concentration indicate both strains to share a very similar copper metabolism. All the data presented are consistent with a detoxification function of the low- M_r copper-binding fraction of *N. crassa*. Both copper-metallothionein and oxidized glutathione (GSSG) are co-eluted with this fraction. The possible involvement of glutathione in metallothionein biosynthesis is discussed.

INTRODUCTION

The toxicity of copper towards fungi is well known and has been studied by many authors (e.g. Martin *et al.*, 1942; McCallan, 1949). The fungicidal effects of copper were shown to depend on the selective accumulation of this metal ion by fungal spores (Sisler & Cox, 1960; Somers, 1962). Moreover, fungal spores have been demonstrated to accumulate copper in very large amounts, even at concentrations exceeding that in the external medium (McCallan & Miller, 1958). So far, however, the nature of the copper-uptake process by fungal cells has not been investigated in great detail, and only a few reports have been published on this subject. Lowry *et al.* (1957) and Somers (1963) proposed two contradictory models of the mechanism of copper accumulation by *Neurospora crassa*. Whereas Lowry *et al.* (1957) postulated that the copper uptake by *N. crassa* represents a process of adsorption to the cell wall, Somers (1963) found the *N. crassa* cell wall to show relatively low affinity towards copper. He considered the initial event of copper uptake to be an ion-exchange reaction at the cell wall, followed by permeation of the metal through the cell membrane.

The fact that very large amounts of copper are required to produce toxic effects in *N. crassa* (McCallan & Miller, 1958; Somers, 1963) suggests the presence of an efficient detoxification system in this organism. Both the intracellular reduction of the cupric ion to the less toxic cuprous form (Tröger, 1956) and 'antidoting' by metabolic agents inside the cell wall (Adam & Powell, 1957) have been proposed as possible detoxification mechanisms in fungi. In eukaryotic organisms, heavy metals are known to induce the synthesis of a set of proteins, including metallothioneins (for a review, see Kägi *et al.*, 1984), that sequester large amounts of this

metal ion. It was shown by Lerch (1980) that, in *N. crassa*, accumulation of copper leads to the concomitant synthesis of a low- M_r copper-binding protein, which was classified as metallothionein on the basis of its strong structural relationship to the mammalian metallothioneins.

In the present study we report some new aspects on the mode of copper accumulation by *N. crassa*. The comparison of the copper uptake by a cell-wall-deficient mutant with a wild-type strain revealed more insight into the role of the cell wall in copper trapping. Furthermore, the intracellular distribution of the metal ions accumulated was investigated under different extracellular-copper conditions. These data provide some new aspects on metallothionein biosynthesis in *N. crassa*.

MATERIALS AND METHODS

Fungal strains and culture conditions

N. crassa wild-type strain Sing T-120-1 was obtained from Dr. N. H. Horowitz (California Institute of Technology, Pasadena, CA, U.S.A.). *N. crassa* slime variant FGSC #1118 was obtained from Fungal Genetics Stock Center, Kansas City, KS, U.S.A.

N. crassa wild-type strain and *N. crassa* slime variant were cultured in 1-litre Erlenmeyer flasks (with intrusions for cells with hyphal growth) containing 200 ml of medium. The medium was essentially as described by Vogel (1956). Slime cultures were osmotically stabilized by addition of 0.25 M-sorbitol. Copper was added to the culture medium in the form of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The cultures were inoculated at an initial density of about 10^8 cells/ml. The flasks were agitated on a rotatory shaker (100 rev./min) at 25 °C in the dark. Wild-type cells were harvested by filtration through cheesecloth and sub-

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sequently freeze-dried. Slime cells were separated from the medium by a simplified version of the method of Scarborough (1975). The cells were centrifuged at 650 *g* for 10 min and washed twice with ice-cold buffer (50 mM-Tris/HCl/10 mM-MgSO₄/0.25 M-mannitol, pH 7.5). The final pellet was either immediately extracted or stored at -80 °C.

Preparation and gel filtration of crude extracts and isolation of copper-metallothionein

Freeze-dried mycelium (0.5 g) was pulverized in a Waring Blender and extracted by stirring in 10 ml of 0.1 M-sodium phosphate, pH 7.2, at 4 °C for 10 min. The suspension was centrifuged at 48000 *g* for 10 min and the cytosolic supernatant was applied on a column (2.5 cm × 40 cm) of Sephadex G-50 (Pharmacia) in 10 mM-Tris/HCl, pH 8.0. Slime cells obtained from a 200 ml culture were ruptured by osmotic shock in 8 ml of water on a vortex mixer. After the addition of 2 ml of 0.5 M-sodium phosphate, pH 7.2, extraction and gel filtration were performed as described above.

Copper-metallothionein was isolated under anaerobic conditions by a combination of gel filtration and ion-exchange chromatography as described by Lerch (1980).

Analytical methods

Copper analyses were performed by atomic-absorption spectroscopy on an Instrumentation Inc. IL 157 instrument employing an air/acetylene flame.

Amino acid analyses were carried out on a Durrum D-500 amino acid analyser after sample hydrolysis in 6 M-HCl *in vacuo* at 110 °C for 22 h. For the determination of cysteine, samples were oxidized with performic acid (Hirs, 1967) before hydrolysis. Values given in Table 2 (below) are corrected for the partial loss of serine during the hydrolytic procedure.

For h.p.l.c. analysis, samples were applied on a LiChrosorb RP-18 reversed-phase column. The buffer systems used was buffer A [10 mM-sodium perchlorate in 0.1% (v/v) phosphoric acid, pH 2.1] and buffer B [same as A, but containing 60% (v/v) acetonitrile].

Chromatography was carried out at 20 °C by using a linear gradient of buffer B (0.33%/min) with a flow rate of 1 ml/min. Absorbance was recorded at 220 nm.

RESULTS

Copper-uptake kinetics

The cell-wall-less slime mutant and the wild-type strain were both grown in liquid cultures supplemented with 0.5 mM-CuSO₄. At different times after inoculation the increase of cell mass and the copper content of the media were determined. The results are presented in Fig. 1. In both strains, copper is taken up during the exponential growth period, starting about 30 h after inoculation and exhibiting saturation kinetics, with a constant level reached after 70 h growth time. The slime mutant (Fig. 1a), however, accumulates less than half of the amount of copper (23% of initial copper in the culture medium) as compared with the wild-type strain (51%; Fig. 1b).

Intracellular copper distribution

The copper distribution in the cytosolic 48000 *g* supernatant was analysed by size fractionation on Sephadex G-50. As shown in Fig. 2, depending on the

Fig. 1. Growth curve of *N. crassa* slime variant (a) and wild-type strain (b) in the presence of 0.5 mM-CuSO₄.

The production of cell mass (▲) and the loss of copper ions from the medium (●) are shown as a function of time.

Fig. 2. Sephadex G-50 elution profiles of crude extracts from *N. crassa* slime variant (a) and wild-type strain (b).

For technical details see the Materials and methods section. Cultures were grown for 72 h in the presence of 0.5 mM-CuSO₄ (●) and 3.5 mM-CuSO₄ (▲) respectively.

Table 1. Intracellular distribution of copper ions in *N. crassa*

The copper content of three cytosolic copper-binding fractions obtained by gel filtration (see Fig. 2) was determined by atomic-absorption spectroscopy. *N. crassa* (cell-wall-less) slime variant and wild-type strain were harvested after 72 h growth time. Abbreviation: n.d., not determined.

Strain	[Copper] in the medium		Total copper accumulated (mg/l)	Cytosolic copper content (mg/l)	Relative amounts of copper (%)		
	(mM)	(mg/l)			High- M_r copper compound	Low- M_r copper compound	Free ionic copper
Slime	0.5	31.8	7.2	1.1	71	15	14
	3.5	222.6	n.d.	2.2	63	29	8
Wild-type	0.5	31.8	16.2	5.0	55	45	—
	3.5	222.6	n.d.	11.8	26	69	5

copper concentration in the culture medium, three different copper-containing fractions are apparent for both strains: (i) a high- M_r copper-binding fraction eluted at V_0 on the column, (ii) a low- M_r (3000) copper-binding fraction and (iii) free ionic copper eluted at V_i on the column. The distribution of intracellular copper between these three fractions for both strains grown at two different copper concentrations (0.5 mM and 3.5 mM) is summarized in Table 1. Most interesting, the relative copper content of the low- M_r copper-containing fraction is markedly increased at the higher exogenous copper concentration in the culture medium.

Influence of the copper concentration in the culture medium

Both wild-type strain and slime mutant were grown for a constant time (72 h) at various exogenous copper concentrations. The effects on growth, intracellular copper accumulation and cytosolic copper distribution are shown in Fig. 3. Only the curves describing the total intracellular copper accumulation differ markedly. Copper import into the cytosol of the slime variant (Fig. 3a) fits the equation of Michaelis & Menten (1913) and is characterized by a K_m of 1.45×10^{-3} M. The corresponding curve measured for the wild-type strain, however, appears to be biphasic, with a characteristic burst phase at low, non-saturating, copper concentrations in the culture medium (0–0.5 mM), followed by a slow, but continuously increasing, copper import at higher, non-toxic, copper concentrations (0.5–4 mM).

Alternatively, cultures of both strains were grown at the limiting non-toxic copper concentration of 3.5 mM in the culture medium, and copper uptake, cell-mass production and the distribution of cytosolic copper were analysed at various times after inoculation. These results are presented in Fig. 4.

H.p.l.c. of the low- M_r copper-binding fraction

Copper-metalllothionein was reported (Lerch, 1980) to be co-eluted with the low- M_r copper-binding fraction obtained by gel filtration of crude extracts of *N. crassa*. Rechromatography of low- M_r copper-binding pools of both the slime variant and the wild-type strain on the h.p.l.c. column revealed not only the presence of copper metalllothionein, but also that of GSSG (Figs. 5a and 5b). These components were identified both by the com-

Fig. 3. Effect of the copper concentration in the culture medium on cell-mass production ($\blacktriangle$), total cytosolic copper content ($\bullet$), the amounts of copper of the high- ($\triangle$) and low- ($\circ$) M_r copper-binding fractions and the freely available ionic copper ($\square$) in *N. crassa* slime variant (a) and wild-type strain (b)

Cells were harvested after 72 h growth time. The three cytosolic copper-binding fractions shown were separated by gel filtration as described for Fig. 2.

parison of their elution behaviour with authentic probes (Fig. 5c) and amino acid analysis after performic acid oxidation (Table 2).

The analysis of the relative amounts of copper metalllothionein and oxidized glutathione synthesized by the wild-type strain at different growth times is shown in Fig. 6. Cell cultures were supplemented with 0.5 mM- CuSO_4 (Fig. 6a) and 3.5 mM- CuSO_4 (Fig. 6b) respectively.

Fig. 4. Growth curves of *N. crassa* slime variant (a) and wild-type strain (b) in the presence of 3.5 mM- CuSO_4 in the culture medium

The increase of cell mass ($\blacktriangle$), the amounts of copper corresponding to the high- ($\triangle$) and low- ($\circ$) M_r copper fractions and the freely available ionic copper ($\square$) were measured as a function of time. The size fractionation of the three cytosolic copper-containing fractions shown is presented in Fig. 2.

Table 2. Amino acid composition of copper-metallothionein (Cu-MT) and GSSG isolated by h.p.l.c. of low- M_r copper-binding pools of *N. crassa* slime variant (see Fig. 5a)

Amino acid	Composition (residues/molecule)	
	Cu-MT	GSSG
Cys*	6.6	0.9
Asx	3.2	—
Glx	—	1.2
Ser†	6.5	—
Gly	6.0	1.0
Ala	1.0	—
Lys	1.0	—

* Determined as cysteic acid as described by Hirs (1967).

† Extrapolated to zero time of hydrolysis.

DISCUSSION

In the present study we compare the effect of copper added to the culture medium on growth, extent of uptake and intracellular distribution of this metal ion in the cell-wall-deficient slime variant of *N. crassa* with a wild-type strain. Most of the copper is taken up from the culture medium during the exponential growth period of the organisms (Fig. 1). The cell-wall-less slime mutant, however, accumulates markedly less copper than the wild-type strain. The slime culture medium, which additionally contains sorbitol as an osmotically stabilizing agent, was not found to affect the copper uptake by the wild-type strain (results not shown). Furthermore, other *N. crassa* hyphal-growing strains tested [e.g. FGSC # 327 (Fungal Genetics Stock Center, Kansas City, KS, U.S.A.), the heterokaryon from which the slime variant was selected] accumulated virtually the same amounts of copper as the wild-type strain described (results not

Fig. 5. H.p.l.c. elution profiles obtained from the low- M_r copper-binding pools of *N. crassa* slime variant (a) and wild-type strain (b)

The organisms were grown on medium supplemented with 3.5 mM- CuSO_4 for 72 h. The low- M_r copper-binding fraction was isolated by gel filtration of crude extracts as described in Fig. 2. In (c) the elution behaviour of authentic standards [GSH (Fluka), GSSG (Fluka) and Cu-MT (copper-metallothionein isolated from *N. crassa*; Lerch, 1980)] is presented.

Fig. 6. Analysis of the relative amounts of copper metallothionein (Cu-MT) and GSSG synthesized by *N. crassa* wild-type strain

Mycelia were grown in the presence of 0.5 mM- CuSO_4 (a) or 3.5 mM- CuSO_4 (b) and harvested at different growth times (16, 24 and 72 h). The low- M_r copper-binding pools obtained by gel filtration of crude extracts (Fig. 2) were rechromatographed by h.p.l.c. as described in the Materials and methods section.

shown). Hence it can be concluded from our data that the cell wall of *N. crassa* causes an efficient copper uptake. The outer surface of fungal cells was shown to be negatively charged at neutral pH (Douglas *et al.*, 1959) and may therefore interact electrostatically with copper ions. Furthermore the cell wall of *N. crassa* mainly consists of polysaccharides such as chitin, β 1,3- and β 1,6-glucans (Deacon, 1980), molecules that are known to chelate considerable amounts of metal ions (Muzzarelli & Tanfani, 1982).

The analysis of the copper content in the cytosolic 48000 *g* supernatant (Table 1) indicates that, in both the cell-wall-less slime mutant and the wild-type strain, substantial amounts of the copper taken up are bound to subcellular structures (e.g. the plasma membrane). Moreover, large amounts of copper may be attached to the cell wall of the hyphal-growing strain.

To compare further the mechanisms of copper uptake of the two strains investigated, the amounts of copper in the cytosolic fraction were monitored as a function of various concentrations of this metal ion in the growth medium (Fig. 3). In both strains the intracellular-copper-uptake curves clearly show that free diffusion can not be the underlying mechanism of copper import. The curve describing the intracellular copper accumulation in the slime cells (Fig. 3a) suggests that the import of copper ions into the cytosol either is mediated by an active transport system located within the cell membrane or is achieved by facilitated diffusion. The corresponding curve for the wild-type strain (Fig. 3b) suggests the copper-uptake process to be much more complex. The

results may be interpreted by assuming the presence of a metal-binding component within the cell wall that controls subsequent permeation of the copper ions through the cell membrane. This mechanism would also be consistent with the fact that, in fungal spores, the intracellular copper concentration may extensively exceed the exogenous copper concentration in the culture medium (McCallan & Miller, 1958). At high copper concentrations in the growth medium, both transport systems described may also act as protection mechanisms against the toxic effects of copper ions. They must be equally effective, as both strains survive copper concentrations of up to 4.5 mM (Fig. 3) in the citrate-containing media.

Taking into account the different amounts and mechanisms of copper uptake, we decided to investigate whether the two strains also differ in the biosynthesis of copper proteins, especially of metallothionein. By gel filtration of crude extracts, three cytosolic copper-containing fractions were separated (Fig. 2). As shown in Fig. 3, in both types of *N. crassa* cells (harvested at the stationary growth phase), only the low- M_r copper-binding fraction increases continuously as a function of the exogenous copper concentration, whereas the high- M_r copper-containing fraction reaches a saturation value. As a consequence, we suggest that the low- M_r copper-binding fraction is responsible for the intracellular detoxification of copper ions. This conclusion is further corroborated by the fact that, at very high extracellular copper concentrations in the culture medium, this fraction decreases, when toxic effects are manifested by a loss of cell mass and an increase of intracellular free ionic copper (Fig. 3). Moreover, the hypothesis is further supported by the growth curves for the two *N. crassa* strains at high, but non-toxic, copper levels (Fig. 4). Regardless the fact that the two morphologically different *N. crassa* strains appear to use separate ways to accumulate copper, they seem to possess a very similar copper metabolism. In the slime variant, however, both the biosynthesis and degradation of the copper-binding fractions are retarded compared with the wild-type strain. Particularly noteworthy is that, in both strains, free ionic copper appears intracellularly about 30 h after inoculation. The decrease in this freely available cytosolic copper is accompanied by a second increase in the low- M_r copper-binding fraction. Again these data are in agreement with a detoxification function of this fraction. The fact that free ionic copper is present for a rather long time could account for a very slow induction of biosynthesis of this detoxification compound.

To analyse further the composition of the low- M_r copper-binding fraction, h.p.l.c. was used (Fig. 5). In each examined *N. crassa* culture grown in the presence of 3.5 mM- CuSO_4 in the culture medium, copper-metallothionein and also, surprisingly, GSSG were detected. As was shown in a control experiment, GSSG and copper-metallothionein are not eluted at the same volume from Sephadex G-50 (results not shown). Hence it appears that GSSG in the low- M_r copper-binding fraction either is associated with copper-metallothionein or originates from a copper-glutathione complex of similar M_r . The fact that substantial amounts of copper ions were always detected in the breakthrough fraction of the h.p.l.c. column (results not shown) rather argues for the presence of copper-glutathione complexes. The

ratio between metallothionein and GSSG in the low- M_r copper-binding fraction seems to depend on the growth time (Fig. 6). An accurate quantitative analysis is rather difficult; nevertheless, it is obvious that, in the wild-type cells, GSSG is present from the beginning of metallothionein biosynthesis. All these findings suggest that, in *N. crassa*, glutathione might play an important role in metallothionein biosynthesis. In contrast with mammalian metallothioneins, which are able to bind various heavy-metal ions (for a review see Kägi *et al.*, 1984), *N. crassa* metallothionein contains exclusively copper. Recent chemical and spectroscopic studies showed the copper ions (6 g-atoms per molecule) to be bound in the form of a single Cu(I)-thiolate complex to cysteine residues (Beltramini & Lerch, 1983a). Hence, intracellularly free available cupric ions must be reduced to cuprous ions to build up the metal cluster. It is possible that glutathione is involved in this step and could subsequently provide the cuprous ions to apo-thionein. Complexes of Cu(I)-glutathione have been obtained *in vitro* and were structurally and spectroscopically characterized by Anglin *et al.* (1971). Whether these low- M_r Cu(I)-thiolate complexes occur *in vivo* is, however, not known.

The data presented here suggest that the low- M_r copper-binding fraction is responsible for intracellular copper detoxification in *N. crassa*. Both metallothionein and GSSG are co-eluted with this fraction and are possible candidates for this role. Multifunctional properties of glutathione have been discussed (for a review, see Meister & Anderson, 1983), among these the protection of cells against reactive oxygen compounds and free radicals. The idea that the ubiquitous tripeptide also participates in copper detoxification has not hitherto been considered. The biological significance of the second candidate, copper-metallothionein, is still a matter of debate. Different functions, such as copper detoxification, copper storage, copper transport or metal donation to other copper-requiring molecules, have been proposed (Beltramini & Lerch, 1983b). Recently, the structural gene encoding *N. crassa* copper-metallothionein has been cloned (Münger *et al.*, 1985), thus permitting a study of the regulation of its expression *in vivo* (Münger *et al.*, 1987). The rapid induction of both metallothionein mRNA and native metallothionein observed after administration of a large copper dose to copper-free cultured cells in the exponential growth phase pointed to a transcriptional regulation of metallothionein biosynthesis. Furthermore, the rapid decay of the mRNA level to its initial value and the remarkable stability of the

synthesized protein suggested an important role of copper-metallothionein in the defence of the fungus against this metal ion.

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