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Cobalt resistance in *Neurospora crassa*: overproduction of a cobaltoprotein in a resistant strain

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A cobalt-resistant strain of *Neurospora crassa* (*cor*) is 20-fold more resistant to Co^{2+} when compared with the wild type. DEAE-cellulose and metal-chelate affinity chromatography of cell-free extracts separated cobalt into protein-bound and free ionic fractions. In *N. crassa cor* about 80% cobalt of cell-free extracts was protein bound while the same in wild type was only 25%. Cobalt content of the protein-bound fraction increased with time and cobalt concentration in the growth medium, and was not influenced by related metal ions. A cobaltoprotein (CBP) which is overproduced in *N. crassa cor* and constitutes up to 12% of total protein of extracts was purified. CPB is a brown coloured (absorption peaks at 275, 350 and 440nm), small molecular weight glycoprotein (Mr 8200 daltons) with 28 – 30% carbohydrate (mannose). CBP has 70 μg cobalt mg^{-1} protein. Cysteine, glycine, glutamic acid and aspartic acid are the major amino acid constituents. The role of CBP in cobalt resistance is discussed in relation to other known metalloproteins involved in resistance.

Keywords: cobalt-binding, cobaltoprotein, cobalt-resistant, metal-resistant, *N. crassa*

Introduction

Metal toxicities were first investigated in *Neurospora crassa* by Healy *et al*. (1955), who reported that cobalt toxicity results in 'conditioned iron deficiency' which is manifested by a general decrease in iron-dependent enzymes. Later studies confirmed the same, and also showed that an iron-binding siderophore is excreted both in iron deficiency and in cobalt toxicity (Padmanaban & Sharma 1964). Cobalt toxicity was also shown to interfere with the heme biosynthetic pathway by inhibiting the rate limiting enzyme δ-aminolevulinic acid dehydratase (Padmanaban & Sharma 1966). Excess iron was shown to reverse all the above toxic effects due to cobalt in *N. crassa*.

Continuous exposure of *N. crassa* to cobalt and nickel ions was shown to result in stable resistant mutants, which do not revert back to sensitivity when cultured on metal-free medium (Maruthi Mohan & Sastry 1983, Rama Rao *et al*. 1997). Metal-resistant strains of fungi are highly desirable due to their utility in metal removal from toxic effluents. Both nickel and cobalt-resistant strains of *N. crassa* were shown to have superior potential in removing toxic metal ions from aqueous media (Kumar *et al*. 1992, Karna *et al*. 1996). The first cobalt-resistant *N. crassa* was shown to be 10-fold more resistant to cobalt and nickel when compared with the wild type (Venkateswerlu & Sastry 1973). Cobalt transport, which is energy-dependent in the wild type, was by a passive mode in the resistant strain (Venkateswerlu & Sastry 1970, 1979). Since iron does not reverse growth inhibition due to cobalt toxicity in the resistant strain (it does in wild type), an alternation in iron utilization pathways was suggested to be responsible for resistance. More recently, a cobalt-resistant *N. crassa* (*cor*) non-identical to the above described strain was characterized and resistance was mapped to linkage group IIIR (Wilson *et al*. 1992). This strain was found to have a partial transport block for cobalt, which was due

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to decreased binding to cell surface and isolated cell walls when compared with wild type *N. crassa* (Sajani & Maruthi Mohan 1997). In the present study *N. crassa cor* was used to study the intracellular mechanism of cobalt resistance.

On exposure to metal ions, fungi are known to elaborate a variety of proteins which sequester metal ions. These include metallothioneins (MTs) and other unrelated high molecular weight proteins. Metallothioneins are ubiquitous low molecular weight, heat stable proteins (Hamer 1986). MTs are characterized by high cysteine content and the absence of aromatic amino acids. They have been grouped into three classes (Rauser 1990). Metallothioneins are thought to be involved mainly in metal ion detoxification, storage of metal ions and protection of organisms against ionizing radiation (Karin 1985). In *Neurospora crassa* a copper metallothionein (Cu-MT) has been characterized by Lerch (Lerch 1980). However in copper-resistant isolates of *N. crassa* there was no increased production of Cu-MT nor was the gene for the same amplified (cited in Munger *et al.* 1987) as observed in the case of the yeast system (Mehra & Winge 1991). Though cadmium-induced phytochelatin was also characterized in *N. crassa*, its role in resistance was not studied (Kneer *et al.* 1992).

The terms tolerance and resistance have often been used interchangeably in the literature. In a recent review Gadd (Gadd 1993) suggested the following definitions; 'metal-resistance' is the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned and 'metal-tolerance' is the ability of an organism to survive metal toxicity by means of intrinsic properties and/or environmental modifications of toxicity. In the present paper the term 'resistance' is used to compare the characteristics of the cobalt-resistant strain (*cor*) with that of wild type *N. crassa*. The objective of the present work is to understand the mechanism of cobalt resistance in relation to a specific metal-binding protein.

Materials and methods

Strains, media and growth conditions

Neurospora crassa FGSC# 4200 and the cobalt-resistant strain (*cor* FGSC #7290) were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ±1°C. The general growth procedures and medium composition were similar to those described in earlier studies (Venkateswerlu & Sastry 1970; Maruthi Mohan & Sastry 1983).

Metal analysis

Metal content of mycelia and cell-free extracts was determined following wet acid digestion (Venkateswerlu & Sastry 1970) by atomic absorption spectrophotometry (AAS, Perkin-Elmer 2380).

DEAE-cellulose and metal-chelate affinity chromatography of cell-free extracts

After growth, mycelia from *N. crassa* wild type and the cobalt-resistant strain were harvested, washed thoroughly with distilled water and homogenized with an equal weight of acid-washed sand in 10 volumes (w/v) of Tris buffer (50 mM, pH 6.5). In each case the homogenate was centrifuged for 5 min at 600 *g* to remove sand and debris and the supernatant was centrifuged at 15 000 *g* for 30 min. Cobalt content of the 15 000 *g* supernatant and the pellet was analysed. An aliquot of this supernatant (20 mg protein equivalent) was loaded on a DEAE-cellulose column (7.5 ml bed volume) preequilibrated with 50 mM Tris buffer, pH 6.5. The DEAE-cellulose column was washed with three bed volumes of the equilibration buffer and bound proteins were eluted with a linear gradient of NaCl (0–1 M) in the same buffer at a flow rate of 1ml min^{-1}. Fractions (1 ml) were collected and monitored for protein at 280 nm and cobalt was estimated, after acid digestion, using AAS. The protein content of the 15 000 *g* supernatant was determined with bovine serum albumin as standard by the method of Lowry *et al.* (1951). In other experiments, preformed mycelial mats (72 h) were suspended in 20 ml basal medium containing cobalt ions, incubated for the required time periods in a rotary shaker (100 rpm) incubator at 28°C and processed as described above.

To determine the concentration of free ionic and protein-bound cobalt in cell-free extracts, an aliquot (equivalent to 100 μg cobalt) was loaded on a metal-chelate affinity column (Sepharose 4B immino-di-acetic acid, Sigma Chemical Co., St. Louis, MO; 10 ml bed volume), preequilibrated with 50 mM Tris buffer, pH 6.5. Free ionic cobalt binds to this affinity matrix and was eluted with 10 mM EDTA in the same buffer. Protein-bound cobalt appears in the flowthrough fraction. Cobalt contents of these fractions were determined by AAS.

To determine whether the distribution of cobalt between ionic and protein-bound fractions was an artefact of the fractionation procedures employed, a control experiment was performed as follows: *N. crassa cor* was grown in the absence of cobalt for 72 h and cobalt (400 μg) was added to the mycelial mats during homogenization and processed on DEAE-cellulose/metal-chelate affinity columns as described above.

Purification of cobaltoprotein

Step 1. Growth and extraction. *N. crassa cor* was grown in the presena of 8 mM $CoSO_4$ for 72 h at 28 °C. The mycelia pooled from 20 flasks (2 g fresh weight) were washed with distilled water and homogenized with acid-washed sand in 20 volumes [w/v] of Tris buffer (50 mM, pH 6.5). The

homogenate was centrifuged at 600 *g* for 5 min to pellet out cell debris and sand. The supernatant was further centrifuged at 15 000 *g* for 30 min. The pellet was rehomogenized in 10–15 ml buffer and centrifugation was repeated. The supernatants were pooled.

Step 2. Heat treatment. The clear supernatant from Step 1 was gently stirred for 10 min at 60°C. The resulting precipitate was then removed by centrifugation for 20 min at 15 000 *g*.

Step 3. DEAE-cellulose. The supernatant from Step 2 was applied on to a DEAE-cellulose column (30 ml bed volume) preequilibrated with 50 mM Tris buffer, pH 6.5. The column was washed with 90 ml of this buffer and the bound proteins were eluted with a linear gradient of NaCl (0–1 M) in the same buffer. Fractions (3 ml) were collected and cobalt-containing fractions were pooled.

Step 4. Sephadex G-50. The cobalt-containing fraction from the above step was dialysed and lyophilized to concentrate, and applied to a column of Sephadex G-50 (1 × 90 cm) preequilibrated with 50 mM Tris–HCl, pH 6.5, containing 0.1 M NaCl. The column was developed at a flow rate of 30 ml h^{-1} and fractions (2 ml) were collected and monitored at 280 nm. The cobalt-containing peak fractions were pooled, dialysed and lyophilized.

Determination of molecular weight

Molecular weight of the purified cobaltoprotein was determined by gel filtration on a Superose-12 column by FPLC (Pharmacia, Bangalore, India). The column was equilibrated with buffer A (50 mM Tris, pH 6.5, containing 0.1 M NaCl) at a flow rate of 24 ml h^{-1}. The void volume (V_0) was determined using blue dextran and the column was calibrated with the following molecular weight markers: BSA (66.7 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa) and cyanacobalamine (vitamin B_{12}, 660 Da).

Absorption spectra of cobaltoprotein were recorded on a Beckmann DU-6 spectrophotometer.

Amino acid and carbohydrate analysis

CBP was hydrolysed (in 6 N HCl at 100°C for 24 h) and the amino acid composition was determined on a Pharmacia LKB alpha plus amino acid analyser calibrated with standard amino acids. Cysteine (Theodore *et al*. 1984), tyrosine (Uhera 1970), tryptophan (Spies & Chamber 1949) and lysine (Shashidhar *et al*. 1994) were determined in intact protein samples by colorimetric methods. Carbohydrate content of cobaltoprotein was determined by the phenol–sulphuric method (Dubios *et al*. 1956).

Results

Cobalt toxicity in wild type and N. crassa cor

Cobalt resistance of *N. crassa cor* was quantitated by determining the IC_{50} (50% growth inhibitory concentration) for cobalt and comparing it with the wild type. The data in Table 1 show that the IC_{50} value of *N. crassa cor* is 20-fold higher (8 mM) when compared with the wild type (0.4 mM). Cobalt uptake determined under the above conditions indicates that the *cor* strain accumulates lower levels of cobalt at 0.4 mM cobalt in the growth medium, whereas it accumulated ten-fold higher concentrations of the metal in their mycelia at 8 mM is accumulated a 10-fold higher concentrations of cobalt in their mycelia. From the above experiment it is apparent that the cobalt-resistant strain not only survives high concentrations of cobalt in the growth medium but also accumulates higher concentrations of cobalt when compared with the sensitive wild type. Hence, fractionation of cobalt from cell-free extracts was undertaken to see whether cobalt was bound to any specific protein.

Major fraction of mycelial cobalt is protein bound in Neurospora crassa cor

DEAE-cellulose chromatography of cell-free extracts of the wild type and the *cor* strain grown at their respective IC_{50} cobalt concentrations (0.4 mM and 8 mM) indicated that in wild type *N. crassa* most of the cobalt (> 70%) was found in the flowthrough fraction and the rest in the DEAE-cellulose bound protein fraction (eluted with salt gradient). In case of the *cor* strain most of the cobalt (> 80%) was located in the protein-bound fraction and very little in the flowthrough fraction (Figure 1). To discount any artefacts and also to see if cobalt-binding protein(s) are present constitutively, cobalt was added to mycelia (*cor* grown in the absence of cobalt) during preparation of cell-free extracts and processed. The data (Figure 1) indicate that most of the cobalt (> 90%) could be accounted for in the flowthrough fraction and was not detectable in the DEAE-cellulose bound protein fraction. This result suggests that

Table 1. Cobalt toxicity in *Neurospora crassa*

	Cobalt (mM)	Growth (mg dry wt)	Cobalt uptake (μg per 100 mg dry wt)
Wild	Nil	42 ± 6	–
	0.4	20 ± 4	28 ± 3
cor	Nil	38 ± 6	–
	0.4	37 ± 4	11 ± 2
	8.0	20 ± 4	257 ± 30

N. crassa strains were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ± 1°C. Cobalt sulphate was included at the required concentrations as indicated. Cobalt was estimated, following acid digestion, by atomic absorption spectrophotometry as described in the text. Values shown are averages from three separate experiments, each in triplicate (± SD).

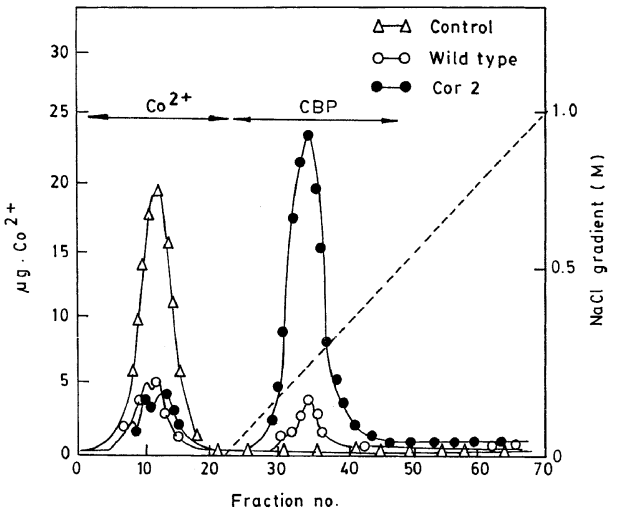

Figure 1. DEAE-cellulose chromatography of mycelial extracts of *N. crassa* extracts. *N. crassa* wild type and *cor* were grown at 0.4 mM and 8.0 mM cobalt for 72 h. The mycelia were processed as described in the text and the supernatant (20 mg protein) was loaded onto a DEAE-cellulose column. For control, 400 μg of cobalt were added to mycelia grown in the absence of cobalt and processed as described above. For details see Materials and methods.

perhaps cobalt-binding proteins(s) are induced in the presence of cobalt.

The cell-free extracts from the above experiments were also analysed by metal-chelate affinity chromatography in which the matrix binds free ions, and protein-bound metals appear in the flowthrough fraction. The data in Table 2 shows that most of the cobalt (80%) from the *cor* strain, grown either in 0.4 mM or 8.0 mM cobalt appears in the flowthrough fraction (protein bound) and the remaining ions (20%) bind to the matrix (eluted with 10 mM EDTA). In case of wild type *N. crassa*, most of the cobalt was found in the ionic form bound to the matrix. In other experiments cobalt appearing in flowthrough fractions of DEAE-cellulose was totally adsorbed to the metal-cheltating matrix, while the flowthrough fraction from the metal-chelate column was totally adsorbed by DEAE-cellulose (data not shown). Both the experiments taken together indicate that the cobalt of cell-free extracts is separable into ionic and protein-bound fractions and that in *N. crassa cor* most of cobalt is protein bound.

The cobalt of the DEAE-cellulose bound protein fraction increases with the cobalt concentration in the growth medium from 2–8 mM. The results presented in Figure 2 indicate that at the relatively non-toxic levels of cobalt in the growth medium (2 mM), most of the cobalt of cell-free extracts of *N. crassa cor* is in a protein-bound fraction and ionic

Table 2. Separation of ionic and protein-bound cobalt by metal-chelate affinity chromatography

	Cobalt (mM)	Cobalt (μg)		
		Cell-free extract	(ionic) bound	(protein bound) flowthrough
Wild	0.4	100	82 ± 8	16 ± 5
cor	0.4	100	5 ± 2	93 ± 6
cor	8.0	100	18 ± 5	80 ± 7
cor	Nil*	100	96 ± 6	ND

N. crassa wild type and *cor* were grown in presence of cobalt for 3 days. Mycelia were pooled and homogenized, and cell-free extracts (15 000 *g* supernatant) containing 100 μg cobalt were loaded onto a metal-chelate affinity column as described in Materials and methods.

*Cobalt added to control mycelia (grown in the absence of cobalt). ND, not detectable. Values shown are from two separate experiments (± SD).

cobalt is not detectable. In the presence of toxic concentrations of cobalt (4–8 mM), ionic cobalt content also increases to a level that is observed with the wild type, implying that perhaps this is the fraction of cobalt which actually causes toxicity. A time course of cobalt distribution into DEAE-cellulose bound protein and flowthrough (ionic) fractions indicated that the cobalt content of the protein-bound fraction in the wild type was more or less similar at both the time points examined (Table 3). Of the total cobalt of the cell-free extract separated on the DEAE-cellulose column, protein-bound cobalt accounted for approximately 28% in the wild type. In the case of

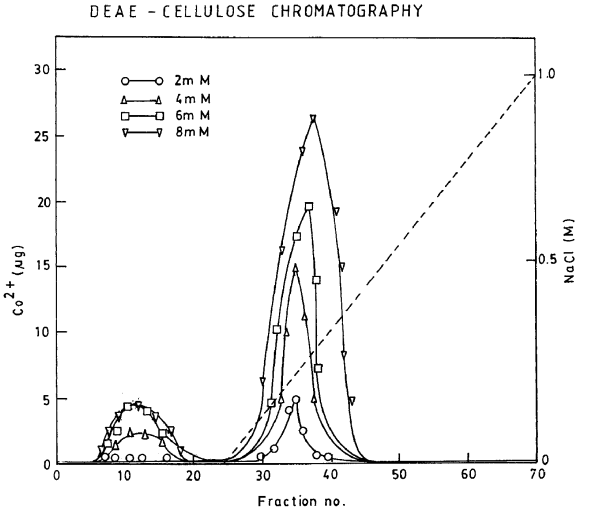

Figure 2. Effect of Co^{2+} concentration on its distribution in mycelial extracts of *N. crassa cor*. DEAE-cellulose chromatography of soluble mycelial extracts (15 000 *g* supernatant) of *N. crassa cor* grown at different concentrations of cobalt. See Figure 1 legend.

Table 3. DEAE-cellulose chromatography of cell-free extracts

	Time (h)	Cobalt (μg) 1. Total loaded	2. Flow-through	3. CBP
Wild type	12	348 (100)	252 (72)	96 (27)
	24	470 (100)	329 (70)	13 (29)
	4	16 (100)	9 (56)	51 (34)
cor	12	193 (100)	82 (42)	104 (54)
	24	440 (100)	80 (20)	308 (77)

Preformed mycelia (72 h) of *N. crassa* strains were floated in 20 ml basal medium containing 6 mM Co^{2+} in 100 ml conical flasks. The mycelial mats were harvested at the indicated time intervals and processed as described in Materials and methods. Representative values of four different experiments are shown (SD up to ± 12%). Values indicated in parentheses are percentages.

1. Soluble protein (20 mg of 15 000 *g* supernatant) was fractionated by DEAE-cellulose column chromatography.

2. DEAE-cellulose flowthrough (three bed volumes) was collected for cobalt estimation.

3. CBP-cobalt binding protein fraction: DEAE-cellulose bound protein was eluted with 0.3 M NaCl in buffer (two bed volumes).

the *cor* strain, the protein-bound cobalt content increased from 34% at 4 h to 77% at 24 h.

In order to see the effect of closely related metal ions on cobalt distribution, zinc, copper, iron and nickel were individually included in the growth medium along with cobalt for 24 h. The results presented in Table 4 show that the presence of other metal ions decreased the overall cobalt uptake (μg 20 mg^{-1} protein), Ni and Zn causing more significant effects.

However, none of the metal ions tested had any significant influence in the distribution pattern of cobalt in protein-bound and ionic fractions, nor were they themselves located in the protein fraction. In each case approximately 90% of the tested metal ions appeared in the DEAE-cellulose flowthrough fraction, suggesting that the cobaltoprotein is induced by cobalt and specifically binds cobalt. In separate experiments Ni, Zn, Cu and Fe individually did not induce metal-binding proteins under the experimental conditions.

Properties of purified cobaltoprotein (CBP)

Since the majority of the cobalt in *N. crassa cor* is associated with the protein fraction, purification of the same was achieved by standard protocols (see Materials and methods) and the results are presented in Table 5. A single cobaltoprotein (CBP) which constitutes about 12% of the total protein of cell-free extracts of *cor* was purified. The purified protein has 70 μg cobalt mg^{-1} protein. Molecular weight analysis by FPLC Superose gel filtration chromatography indicated it to be Mr 8200 daltons. CBP has 28–30% carbohydrate, composed of mannose as the only major sugar residue. The amino acid composition of cysteine (29%), glycine (17%), glutamic acid (15%) and aspartic acid (6%) are calculated taking the total number of nanomoles of all amino acids from Table 6 as 100%. The percentage given for these amino acids is in line withe the same. Aromatic amino acids are also present (tyrosine (6%) and trypto-phan (4%)). Some of the amino acids were also estimated in intact protein colorimetrically (Table 6). Cobaltoprotein is brown coloured with characteristic absorption peaks at 275 and 350 nm, and a shoulder at 440 nm (Figure 3).

Table 4. Effect of metal ions on cobalt distribution between ionic and cobaltoprotein fractions

Metal	Co + M (μg) 1. Total loaded	2. Flowthrough	3. CBP
Co + nil	400	80	308
Co + Zn	133 + 153	29 + 146	101 + ND
Co + Cu	275 + 120	57 + 116	217 + ND
Co + Fe	324 + 158	74 + 145	246 + ND
Co + Ni	128 + 110	26 + 102	99 + ND

Preformed mycelia (72 h) of *N. crassa cor* were incubated in 20 ml basal medium containing 6 mM Co^{2+} and 6 mM each of the metal ions (+ M) indicated for 24 h. Values shown are averages of two separate experiments.

ND, not detectable.

1., 2. and 3. are the same as in Table 3.

Anamolous behaviour of CBP

Separation of CBP by PAGE or SDS–PAGE (15–20% gels) showed the brown coloured CBP band to migrate along the tracking dye, such that it was eas-

Table 5. Purification of cobaltoprotein

Purification step	Protein (mg)	Cobalt (μg)	Specific activity of cobalt (μg mg^{-1} protein)
Crude extract	63	504	8
Heat denaturation	22.40	443	19.8
DEAE-cellulose	9.56	349	36.5
Sephadex G-50	8.40	328	39.0

Table 6. Amino acid composition of cobaltoprotein

Amino acid	*n* moles
A:	
Aspartic acid	5.3
Threonine	0.9
Serine	2.7
Glutamic acid	14.0
Proline	0.77
Glycine	15.9
Alanine	2.6
Iso-leucine	0.5
Leucine	0.7
Tyrosine	7.1
Phenylalanine	0.3
Histidine	2.43
Arginine	0.22
Lysine	1.07
B:	
Cysteine	28
Tryptophan	3.8
Tyrosine	5.8
Lysine	1.4

A: Cobaltoprotein (100 μg) was hydrolysed in 6 N HCl in evacuated tubes at 100°C for 24 h. An aliquot (16 μg) was loaded onto Pharmacia LKB plus amino acid analyser and the concentrations were determined after calibrating the instrument with standard amino acids.

B: Cobaltoprotein (25–250 μg) was used directly in colorimetric procedures as described in Materials and methods. Values shown as *n* moles per 16 μg protein for comparison with values in A.

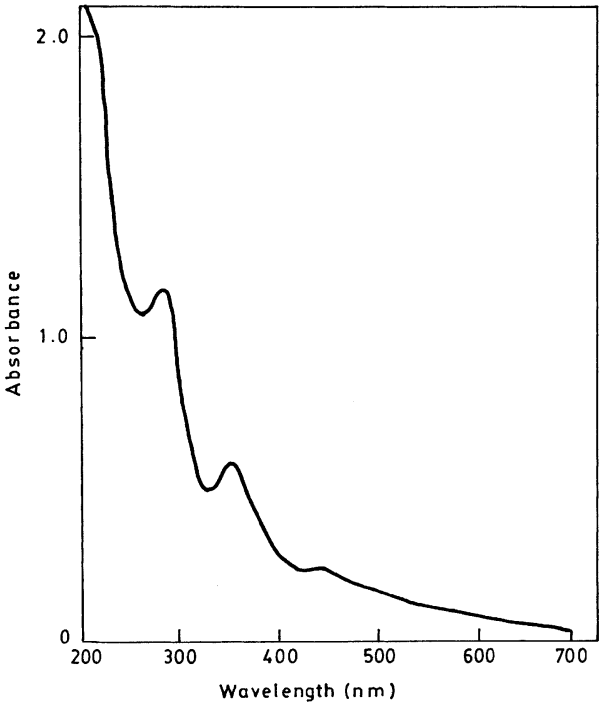

Figure 3. Absorption spectrum of cobaltoprotein.

ily eluted into distilled water. The CBP band could not be fixed by methanol:acetic acid:water (45:10:45). Most of the CBP (approximately 90%) loaded on the above gels could be recovered by elution as monitored by optical density at 280/350 nm and cobalt content. Further staining the gels by sensitive silver staining or Coomassie blue showed no other protein bands, indicating relative purity of CBP preparations. CBP was found to be soluble in 15% trichloroacetic acid. To date, removal of cobalt from CBP by methods employed for metallothioneins have not been successful in our laboratory. CBP was found to be resistant to digestion with pronase.

Discussion

Metal resistance in fungi in general could be due to two broad mechanisms: (i) transport block; or (ii) intracellular sequestration by specific proteins. The latter mechanism is more commonly observed in the yeast and fungal systems (Hamer 1986, Mehra & Winge 1991, Gadd 1993). Metallothioneins are cysteine-rich small molecular weight proteins which have also been characterized in *N. crassa* and a variety of yeast (Nordberg & Kojima 1979, Lerch 1980, Karin 1985, Butt & Ecker 1987). Metallothioneins have been implicated in homeostasis and metal detoxification functions. Copper metallothionein (Cu-MT) of *N. crassa*, a cysteine-rich protein (MW 2200 daltons) containing only seven different amino acids, has been characterized in detail and the gene for Cu-MT has been cloned and sequenced (Lerch 1980, Munger *et al.* 1987). The above studies have suggested a role for this protein in copper homeostasis as a storage protein and in detoxification. However, in copper-resistant *N. crassa* there was no overproduction of Cu-MT nor was the gene for the same was amplified. This is in contrast with the metallothioneins of yeast wherein metal resistance correlated well with gene amplification (Fogel & Welch 1982; Butt & Echker 1987, Mehra *et al.* 1900). Further, in *N. crassa* Cu-MT was not induced by Cd, Co, Ni and Zn (Munger *et al.* 1987). Copper toxicity in *N. crassa* on a sole nitrate nitrogen and inorganic phosphate medium results in blue coloured mycelia and cell walls (Subramanyam *et al.* 1983) and a copper-binding protein has been purified and characterized from blue cell walls (Suresh & Subramanyam 1996). A γ-glytamyl peptide was also shown to be induced in *N. crassa* with cadmium as seen in the case of *S.cerevisiae* (Kneer *et al.* 1992). However, its role in resistance was not studied. Aphosphoglycoprotein-binding cadmium lack-

ing thiol groups was also reported in *Agaricus macrosporus* (Neich *et al*. 1983).

In the present study a novel cobaltoprotein (CBP), a small molecular weight glycoprotein nonidentical to the above described *N. crassa* metal-binding proteins, overexpressed by the cobalt-resistant strain (*cor*) was characterized. Fractionation of cell-free extracts by DEAE-cellulose and metal-chelate affinity chromatography separated cobalt into protein-bound and ionic fractions. Most of the cobalt of *N. crassa cor* was located in the protein-bound fraction, in sharp contrast to that of the parental wild type *N. crassa*, wherein most of the cobalt was observed in the ionic fraction. Further, cobaltoprotein was induced only by cobalt ions in a time-dependent manner in the *cor* strain (Figure 2). Related metal ions (Fe, Zn, Cu and Ni) did not induce this protein nor were they bound to this protein fraction, when included along with cobalt ions. Hence, cobaltoprotein is a cobalt-specific protein and is over expressed only in the *cor* strain, where it accounts for about 12% of the total protein; this clearly points to a detoxification function. Unlike copper, cobalt is not known to be required by *N. crassa* for growth as trace element. Further, a cyanocobalamine-dependent metabolism is absent (Dalal *et al*. 1961) and hence a storage function cannot be envisaged. It is a well known fact that metal ions exert toxicity more in the ionic form than when complexed (Gadd & Griffith 1978), which once again lends support to a detoxification function for cobaltoprotein.

Compositional analysis indicated the presence of a high content of cysteine (29%) and glycine (17%) as in metallothioneins, but with an excess of acidic amino acids (glutamic and aspartic acids) making CBP a highly acidic protein. This property is in fact responsible for the binding of CBP to DEAE-cellulose even at pH 6.5. The presence of aromatic amino acids (tyrosine and tryptophan) and acidic amino acids normally absent in metallothioneins is yet another distinctive feature of cobaltoprotein. In addition, some unassigned peaks were observed which could represent unusual amino acids yet to be identified. The presence of carbohydrate represented by mannose residues accounting for 28–30% of the protein (w/w) is yet another important feature of CBP. The most distinct and advantageous feature of CBP is the characteristic brown colour with absorption peaks accounting for aromatic amino acids at 275 nm and chromophore at 350 nm, and a shoulder at 440 nm. In preliminary experiments the appearance of cobaltoprotein in cell-free extracts could conveniently be followed by measuring absorption at 350 and 440 nm. The anamolous behaviour of CBP on PAGE/SDS–PAGE is not surprising as this has been observed for other metallothioneins (Winge & Brouwer 1986), largely due to excess of thiol groups of cysteine.

To our knowledge there has been no report to date of a cobalt-binding protein that is specifically induced by cobalt ions in metal-resistant fungi. Cobalt resistance in *Aspergillus nidulans* and *S.cerevisiae* was shown to involve mechanisms other than sequestration by metallothioneins (Tomsett *et al*. 1989, Joho *et al*. 1991). In wild type *N. crassa*, Ballentine & Stephens (1951) showed that cobalt (^{60}Co) taken up during growth is fixed more firmly and is not leachable by EDTA when compared with cobalt added to cell-free extracts. However, in this case no protein was characterized. Our studies show for the first time that a cobaltoprotein is produced in *N. crassa cor* in response to cobalt and is involved in resistance.

The present data, taken together with our previous report (Sajani & Maruthi Mohan 1997), show for the first time in fungi that both a transport block and intracellular sequestration mechanisms are operating in a single metal-resistant strain. The same is shown graphically in Figure 4, in which the upper half represents the wild type and the lower half represents the *cor* strain of *N. crassa*.

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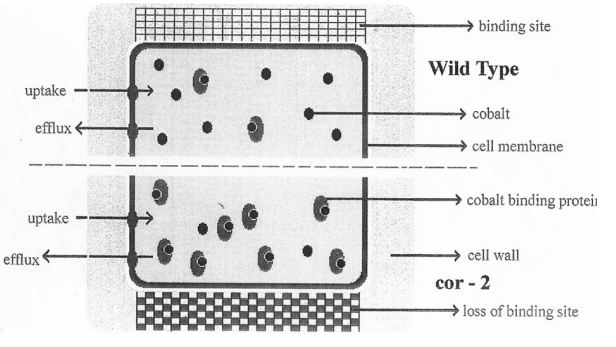

Figure 4. Schematic representation of cobalt resistance in *Neuospora crassa cor*.

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