Further Studies on the Subcellular Distribution of Cd^{2+} in Cd-sensitive and Cd-resistant Strains of *Saccharomyces cerevisiae*

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When a Cd-resistant strain (301N) and a Cd-sensitive strain (101N) of *Saccharomyces cerevisiae* were incubated in medium containing Cd^{2+} , a large proportion of the cellular Cd^{2+} was found in the cytosol of strain 301N, but not in that of strain 101N. Approximately 65% of the cellular Cd^{2+} was released from strain 301N after treatment with chitosan, which affects cell membrane permeability. About 80% of the cellular Cd^{2+} released from strain 301N by chitosan treatment was detected in a 30000–10000 molecular weight fraction prepared by ultrafiltration. The distribution of Cd^{2+} into the cytosol in strain 301N was inhibited in the presence of cycloheximide. The proportion of cellular Cu^{2+} or Zn^{2+} present in the cytosol after incubation with these ions was similar for the two strains (about 40%).

INTRODUCTION

Cadmium is a toxic metal and interacts with many cellular components, resulting in the inhibition of numerous metabolic pathways (Vallee & Ulmer, 1972; Mustafa & Cross, 1971) or of the formation of mitochondria (Lindegren & Lindegren, 1973). It has been reported that in animal and higher plant cells the toxicity of Cd^{2+} is lessened by the synthesis of a low molecular weight protein, metallothionein, which contains a large amount of cysteine (Hildebrand *et al.*, 1979; Enger *et al.*, 1981; Leber & Miya, 1976; Jackson *et al.*, 1984; Beach & Palmiter, 1981). Fogel *et al.* (1983) also demonstrated that the resistance of yeast to Cu^{2+} was correlated with the synthesis of a Cu-chelatin; increased synthesis of this protein resulted from an increased copy number of the DNA at the *CUP1* region.

We have previously reported that when a Cd-resistant strain of *Saccharomyces cerevisiae* is cultured in a medium containing Cd^{2+} , most of the cellular Cd^{2+} is found in the cytosol and bound to protein(s) of low molecular weight (Joho *et al.*, 1985). As a hypothesis for Cd-resistance mechanisms in this Cd-resistant strain, we proposed an increase in the synthesis of Cd-binding protein(s) of low molecular weight. Murasugi *et al.* (1984) reported two kinds of Cd-binding peptides in *Schizosaccharomyces pombe*. It is not clear, however, whether the Cd-binding protein(s) in yeast serve a function in decreasing Cd^{2+} toxicity.

Bracken *et al.* (1984) reported that in bovine kidney cells metallothionein sequestered less than 25% of the cellular Cd^{2+} . If Cd-binding protein(s) in the Cd-resistant strain of *S. cerevisiae* were an effective agent for Cd-detoxification, then large amounts of the cellular Cd^{2+} would presumably be bound to it (them). In the present study, we examined whether Cd-binding protein(s) in the Cd-resistant cells serve as scavengers for cellular Cd^{2+} .

METHODS

Organisms and culture conditions. The Cd-sensitive strain 101N (*MATa, ade7, ura1*) and the Cd-resistant strain 301N (*MATa, ade7, ura1*) of *Saccharomyces cerevisiae* were prepared as described previously (Joho *et al.*, 1985). Cells were grown at 30 °C in a nutrient medium containing: sucrose, 50 g; peptone, 5 g; KH_2PO_4 , 5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g; malt extract (Baumé 8°), 360 ml and distilled water, 1000 ml.

Subcellular fractionation. Cells previously incubated for 3 h at 30 °C in the above medium containing 0.5 mM-

Cd^{2+} were disrupted by a Braun homogenizer in 5 mM-PIPES buffer (pH 6.5) with glass beads. Crude subcellular fractions were obtained by differential centrifugation; the resulting pellets were defined as the 5K pellet (5000 r.p.m., 2000 g, 10 min), the 12K pellet (12000 r.p.m., 11000 g, 30 min), the 36K pellet (36000 r.p.m., 90000 g, 90 min) and the 36K supernatant.

Treatment with chitosan. Cells grown in the nutrient medium at 30 °C for 24 h were harvested and transferred into fresh nutrient medium containing $\text{CdSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ or $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. After incubation at 30 °C, the cells were harvested, washed twice with distilled water and treated with chitosan (50 $\mu\text{g ml}^{-1}$) in 5 mM-PIPES buffer (pH 6.5) at 30 °C. The times of incubation are given in Results.

Fractionation of substances released from cells by chitosan treatment. The supernatant fraction obtained from cells treated with chitosan was fractionated by Centricon ultrafiltration (Amicon). Sequential filtration through Centricon-30 and Centricon-10 membranes fractionated the soluble components into > 30000, 30000–10000 and < 10000 equivalent molecular weight fractions (based on globular proteins).

Assay of Cd^{2+} and Mg^{2+} . Untreated cells, the pellets obtained by differential centrifugation after Braun homogenization, and the pellet (2000 g, 5 min) obtained after chitosan treatment were digested with 0.2 ml 6 M- HNO_3 at 100 °C for 30 min in a water bath and diluted with distilled water. Undigested material was removed by centrifugation and the Cd^{2+} and Mg^{2+} contents of the supernatants were determined by atomic absorption spectrophotometry.

RESULTS

Subcellular distribution of Cd^{2+} and Mg^{2+}

Table 1 shows the subcellular distribution of Cd^{2+} and Mg^{2+} in the Cd-sensitive (101N) and the Cd-resistant (301N) strains of *S. cerevisiae* after incubation at 30 °C for 3 h in medium containing 0.5 mM- Cd^{2+} . Approximately 40% of the Cd^{2+} taken up by strain 101N was found in the 36K supernatant fraction, approximately 20% in both the 5K and the 12K pellets, and approximately 30% in the 36K pellet. In strain 301N approximately 90% of the cellular Cd^{2+} was found in the 36K supernatant fraction. No significant difference was observed in the Mg^{2+} distribution in the fractions obtained from strains 101N and 301N; approximately 60% of the cellular Mg^{2+} was found in the 36K supernatant of both strains.

When cells of *S. cerevisiae* are treated with chitosan, which affects cell membrane permeability, cellular ions and substances of low molecular weight are released from the cells (Joho *et al.*, 1985). Fig. 1 shows the pattern of release of Cd^{2+} and Mg^{2+} from strains 101N and 301N during treatment with chitosan at 30 °C for 60 min. Approximately 65% of the total cellular Cd^{2+} was released from strain 301N, while only 19% was released from strain 101N (Fig. 1*b*). The pattern of release of Mg^{2+} was similar for the two strains, approximately 40% of the total cellular Mg^{2+} being released (Fig. 1*a*). These results indicate that the amount of Cd^{2+} or Mg^{2+} released from the cells after treatment with chitosan is related to the distribution of these ions detected in the 36K supernatant as shown in Table 1.

Table 1. *Distribution of Cd^{2+} and Mg^{2+} in Cd-sensitive (101N) and Cd-resistant (301N) strains of *S. cerevisiae**

After incubation at 30 °C for 3 h in medium containing 0.5 mM- Cd^{2+} , cells (approx. 0.6 g dry wt) were disrupted by a Braun homogenizer with glass beads and fractionated by centrifugation. Values are means from two experiments; the variability between the replicate experiments was within 5% for each fraction.

	Distribution of Cd^{2+}				Distribution of Mg^{2+}			
	101N		301N		101N		301N	
	$\mu\text{g Cd}$	%	$\mu\text{g Cd}$	%	$\mu\text{g Mg}$	%	$\mu\text{g Mg}$	%
Cell homogenate	316	100	148	100	9426	100	8519	100
5K pellet	52	16.5	24	16.2	973	10.3	992	11.6
12K pellet	66	20.8	11	7.4	1429	15.1	745	8.7
36K pellet	87	27.5	7	4.7	905	9.6	830	9.7
36K supernatant	126	39.9	132	89.2	6489	68.8	5519	64.7
Recovery (%)		104.7		117.5		103.8		94.7

Fig. 1. Effect of chitosan on the release of (a) Mg^{2+} and (b) Cd^{2+} from strains 101N (Cd-sensitive; ○) and 301N (Cd-resistant; ●) of *S. cerevisiae*. After incubation at 30 °C for 3 h in medium containing 0.5 mM- Cd^{2+} , cells (approx. 4 mg dry wt ml^{-1}) were treated with chitosan (50 $\mu\text{g ml}^{-1}$) at 30 °C in 5 mM-PIPES buffer (pH 6.5). The release of Mg^{2+} and Cd^{2+} is expressed as $\mu\text{g ion (ml supernatant)}^{-1}$ (—) and as $\mu\text{g ion (mg dry wt pellet)}^{-1}$ (---). Each point is the mean of two determinations; typical results are shown for one of three experiments. The variability among the triplicate experiments was within 5% for each point.

Effect of cycloheximide on the cellular distribution of Cd^{2+}

The uptake of Cd^{2+} increased approximately linearly with increasing Cd^{2+} concentrations in both strain 101N and strain 301N when they were incubated at 30 °C for 3 h in nutrient medium containing various concentrations of Cd^{2+} (Fig. 2a, b). The uptake of Cd^{2+} by strain 301N was 60–70% of that of strain 101N at each concentration of Cd^{2+} tested. In strain 101N, approximately 90% of the total Cd^{2+} remained in the cells after treatment with chitosan, whereas 80% or more of the Cd^{2+} taken up by strain 301N in medium containing 0.25 mM- Cd^{2+} was released from the cells by treatment with chitosan. The pattern of release of Cd^{2+} from strain 301N indicated that a saturation point was reached in medium containing 0.5 mM- Cd^{2+} (Fig. 2b), so that despite further increases in the concentration of Cd^{2+} from 0.5 to 1.0 mM in the medium, the Cd^{2+} level in the fraction released by chitosan remained constant at approximately 0.35 $\mu\text{g (ml supernatant)}^{-1}$.

To determine whether protein synthesis was required for the presence of Cd^{2+} in the cytosol, the cells were incubated with cycloheximide (1 $\mu\text{g ml}^{-1}$) at 30 °C for 1 h before incubation in medium containing various concentrations of Cd^{2+} (Fig. 2c, d). The uptake of Cd^{2+} decreased in the presence of cycloheximide by about 10–30% in strain 101N and 30–40% in strain 301N. In strain 101N, the distribution of Cd^{2+} at varying concentrations was not significantly altered in the presence of cycloheximide (Fig. 2c), whereas in strain 301N the presence of cycloheximide prevented the distribution of Cd^{2+} into the cytosol but not its distribution into the pellet fraction. This indicates that protein synthesis is necessary for the distribution of Cd^{2+} into the cytosol in strain 301N.

Partitioning of Cd^{2+} between the cytosol and pellet fractions

Leber & Miya (1976) reported that in animal cells synthesis of Cd-binding protein was induced by Zn^{2+} . To determine whether the distribution of Cd^{2+} in strain 301N was specific to

Fig. 2. Effect of cycloheximide on Cd^{2+} uptake and distribution in strains 101N (Cd-sensitive) and 301N (Cd-resistant) of *S. cerevisiae*. After incubation at 30 °C for 3 h in medium containing various concentrations of Cd^{2+} in the presence or the absence of cycloheximide ($1 \mu\text{g ml}^{-1}$), cells (approx. 4 mg dry wt ml^{-1}) were treated with chitosan ($50 \mu\text{g ml}^{-1}$) at 30 °C for 1 h in 5 mM-PIPES buffer (pH 6.5). The results are expressed as $\mu\text{g Cd}^{2+} (\text{ml supernatant})^{-1}$ (○---○), as $\mu\text{g ion (mg dry wt pellet)}^{-1}$ (○—○), and as $\mu\text{g ion (mg dry wt cells)}^{-1}$ (●—●). Each point is the mean of two determinations; typical results are shown for one of three experiments. The variability among the triplicate experiments was within 5% for each point.

this ion, we compared the uptake and distribution of Cd^{2+} , Cu^{2+} and Zn^{2+} between the cytosol and the pellet fractions during incubation in nutrient medium containing 0.5 mM concentrations of the respective ions (Fig. 3). Cd^{2+} uptake was rapid during the first hour of incubation after which the rate of uptake decreased. The uptake of Cd^{2+} by strain 101N was higher than that of strain 301N (Fig. 3a, b). In strain 101N, only 13% of the cellular Cd^{2+} was found in the supernatant fraction after 3 h incubation. The level of Cd^{2+} in the cytosol in strain 301N increased with incubation time, whereas the Cd^{2+} content of the pellet fraction did not increase further after 1 h of incubation. This result indicates that Cd^{2+} taken up by strain 301N is preferentially distributed into the cytosol. The uptake of Zn^{2+} by strain 301N was lower than that by strain 101N (Fig. 3c, d), but the amount of Zn^{2+} in the cytosol was similar for the two strains. The uptake of Cu^{2+} by strain 101N was very similar to that of strain 301N (Fig. 3e, f) and, furthermore, about 40% of the total cellular Cu^{2+} was released from both strains.

Molecular weight distribution in the supernatant fraction

In a previous paper, we reported that almost all the Cd^{2+} released from strain 301N by treatment with chitosan was bound to protein(s) of molecular weight of less than 30000 as determined by gel filtration (Joho *et al.*, 1985). The supernatant fractions obtained by treatment with chitosan were further fractionated by ultrafiltration using membranes with equivalent molecular weight exclusion limits of 30000 and 10000. Table 2 shows the distribution of Cd^{2+} , Zn^{2+} and Cu^{2+} in the different molecular weight fractions of the supernatant fraction obtained after treatment with chitosan. (The molecular weight distribution serves only to demonstrate gross differences in the distribution of metal-binding components.) Approximately 80% of the

Fig. 3. Uptake and distribution of Cd^{2+} , Cu^{2+} and Zn^{2+} in strains 101N (Cd-sensitive) and 301N (Cd-resistant) of *S. cerevisiae*. After incubation at 30 °C in medium containing the respective heavy metal (0.5 mM), cells (approx. 4 mg dry wt ml^{-1}) were treated with chitosan (50 $\mu\text{g ml}^{-1}$) at 30 °C for 1 h in 5 mM-PIPES buffer (pH 6.5). The results are expressed as $\mu\text{g ion (ml supernatant)}^{-1}$ ($\bigcirc$ --- $\bigcirc$), as $\mu\text{g ion (mg dry wt pellet)}^{-1}$ ($\bigcirc$ — $\bigcirc$), and as $\mu\text{g ion (mg dry wt cells)}^{-1}$ ($\bullet$ — $\bullet$). Each point is the mean of two determinations; typical results are shown for one of three experiments. The variability among the triplicate experiments was within 5% for each point.

Table 2. Distribution of Cd^{2+} , Cu^{2+} and Zn^{2+} in the fractions obtained by ultrafiltration of supernatants of strains 101N and 301N treated with chitosan

After incubation at 30 °C for 3 h in medium containing Cd^{2+} , Cu^{2+} or Zn^{2+} at a concentration of 0.5 mM, cells (approx. 4 mg dry wt ml^{-1}) were treated with chitosan (50 $\mu\text{g ml}^{-1}$) at 30 °C for 1 h in 5 mM-PIPES buffer (pH 6.5) and centrifuged. The supernatant was fractionated by ultrafiltration. Values are means from two experiments; the variability between the replicate experiments was within 5% for each molecular weight fraction.

Molecular weight fraction	Percentage distribution		
	Cd^{2+}	Cu^{2+}	Zn^{2+}
Strain 101N (Cd-sensitive)			
> 30000	0	10.0	0
30000–10000	37.9	42.0	15.2
< 10000	62.1	48.0	84.8
Metal content*	0.062	0.227	0.217
Strain 301N (Cd-resistant)			
> 30000	0	4.0	0
30000–10000	78.4	53.4	42.6
< 10000	21.6	42.6	57.4
Metal content*	0.268	0.271	0.212

* Amount (μg) in the supernatant solution released from 1 mg dry weight of cells.

Cd^{2+} in the supernatant fraction of strain 301N was found in the 30000–10000 molecular weight fraction, while only about 40% was found in the corresponding fraction in strain 101N. Furthermore, the Cd^{2+} content of the supernatant fraction of strain 301N was some fourfold higher than that in strain 101N. This suggests that protein(s) in the 30000–10000 molecular weight fraction of strain 301N may act as sequestering agents of intracellular Cd^{2+} .

In the supernatant fraction of strain 301N, 43% of the Zn^{2+} was found in the 30000–10000 molecular weight fraction while only 15% was found in the corresponding fraction in strain 101N. The Zn^{2+} content of the supernatant fractions of the two strains was approximately the same, $0.2 \mu\text{g ml}^{-1}$. The higher proportion of Zn^{2+} in the 30000–10000 molecular weight fraction in strain 301N as compared with strain 101N is somewhat similar to the situation observed with Cd^{2+} , and the distribution of Zn^{2+} in strain 301N may therefore be due to a specific Zn^{2+} -binding protein. The content and distribution of Cu^{2+} in the supernatant fraction, however, unlike that of Cd^{2+} and Zn^{2+} , did not differ between the two strains.

Ninety percent or more of the protein content of the supernatant fractions in both strain 101N and strain 301N passed through a Centricon-30 membrane, indicating that the molecular weight of protein(s) released from the cells by treatment with chitosan was less than 30000 (data not shown). Furthermore, approximately 50% of the protein in the supernatant fraction passed through a Centricon-10 membrane (mol. wt cut-off 10000). Therefore, the <10000 molecular weight fraction may contain low molecular weight complexes of peptides or amino acids in addition to inorganic Cd^{2+} , Zn^{2+} or Cu^{2+} (Ramamoorthy & Kushner, 1975).

DISCUSSION

This study supports previous evidence that most of the cellular Cd^{2+} in the Cd-resistant strain 301N is present in the cytosol (Table 1). After treatment with chitosan, 60–70% of the cellular Cd^{2+} was released from strain 301N which had been previously incubated at 30°C for 3 h in medium containing 0.5 mM-Cd^{2+} . Since 80% of the Cd^{2+} in the supernatant fraction from strain 301N after chitosan treatment was detected in the 30000–10000 molecular weight fraction (Table 2), we roughly estimate the Cd-binding capacity of the low molecular weight protein(s) to be at least 50% of the total cellular Cd^{2+} .

The maximum Cd^{2+} content in the cytosol of strain 301N was attained by incubation for 3 h in medium containing 0.5 mM-Cd^{2+} , although the total Cd^{2+} content increased with further increases in the Cd^{2+} concentration of the medium (Fig. 2). The distribution of Cd^{2+} into the cytosol in strain 301N was prevented in the presence of cycloheximide (Fig. 2) and, furthermore, when the time course of the cellular partitioning of Cd^{2+} between the cytosol and the pellet fraction in strain 301N was examined, Cd^{2+} progressively accumulated in the cytosol but not in the pellet fraction (Fig. 3). This progressive accumulation of Cd^{2+} in strain 301N was also prevented in the presence of cycloheximide (data not shown). These results suggest that preferential distribution of Cd^{2+} into the cytosol in strain 301N may be due to Cd-binding protein(s), whose synthesis is induced in the presence of Cd^{2+} (Jackson *et al.*, 1984; Beach & Palmiter, 1981). In contrast to the results with strain 301N, most of the cellular Cd^{2+} in the Cd-sensitive strain 101N was found in the pellet fraction (Fig. 2), indicating that many Cd^{2+} -binding sites exist on the cell membrane or in the cell organelles (Solaiman *et al.*, 1979; Fowler, 1978). Cell membranes and mitochondria contain many enzyme systems which are affected by Cd^{2+} (Nicholls *et al.*, 1981; Vallee & Ulmer, 1972), resulting in the release of K^+ from the cells (Nicholls *et al.*, 1981; Stacey & Klaassen, 1981; Gadd & Mowll, 1983). Furthermore, since 25% or more of the cellular Cd^{2+} in strain 101N was detected in the 36K pellet fraction, which includes ribosomes (Table 1), Cd^{2+} would presumably exert an effect on protein synthesis (Webb, 1979). We therefore suggest that the distribution of cellular Cd^{2+} into the cytosol in strain 301N acts as an effective Cd-resistance mechanism, whereby the cytotoxicity of Cd^{2+} is lowered due to its sequestration by protein(s) of molecular weight 30000–10000. Finally, Cd-binding protein(s) in strain 301N may also be induced by Zn^{2+} , but not, however, by Cu^{2+} (Table 2).

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