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Vanadate and copper induce overlapping oxidative stress responses in the vanadate-tolerant yeast *Hansenula polymorpha*

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Abstract

The mechanisms by which vanadate exerts a toxic effect on living organisms are not completely understood. This is principally due to the variety of intracellular targets of the metal and to the changes in the chemical form and oxidation states that vanadate can undergo, both in the external environment and intracellularly. In order to further elucidate the reasons for vanadate toxicity, and assuming that common detoxification mechanisms can be evoked by a general heavy metal response, we have compared some aspects of the cellular responses to vanadate and copper in the yeast *Hansenula polymorpha*. By means of 2D electrophoresis we show the existence of common determinants in the responses to vanadate- and copper-induced stresses. Moreover, we demonstrate that both metals induce significant increases in antioxidant enzyme levels, and that there are significant overlaps in the heavy metal and oxidative stress responses. Interestingly, vanadate induces an increase in catalase activity that is much higher than that seen with copper and, unlike copper, does not cause lipid peroxidation of cellular membranes. This suggests that *H. polymorpha* cells activate a further specific detoxification pathway against vanadate-induced oxidative insults. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Vanadate toxicity; Heavy metal stress; Oxidative stress; Yeast; 2D electrophoresis; *Hansenula polymorpha*

1. Introduction

The toxicity of heavy metals on living systems is largely mediated by their ability to form coordination complexes and clusters with important cellular targets, such as phosphates, purines, pteridines, porphyrins, cysteinyl and histidyl side chains of proteins [1]. Moreover, heavy metal ions are able to cause oxidative damage either directly, through their redox cycling activities that produce the extremely reactive $OH^{\bullet}$ radical [2], or indirectly, by depleting free radical scavengers such as glutathione and protein bound sulphydryl groups [3].

The yeast *Saccharomyces cerevisiae* responds to the presence of toxic concentrations of copper ions by means of various defense mechanisms, such as an increase in the synthesis of metallothioneins [4,5] and antioxidant enzymes [6,7], and the activation of vacuolar functions [8]. Similarly, vanadate detoxification seems to require the activation of different concerted mechanisms. In the yeast *Hansenula polymorpha*, it has been suggested that vanadate is reduced to the less toxic vanadyl, in which form it can be sequestered by vacuolar polyphosphates [9]. Moreover, during growth on vanadate-containing medium, *H. polymorpha* cells activate an autophagic mechanism which may be needed to compensate for a nutrient depletion and/or eliminate the aberrant cellular structures induced by this metal ion [10].

The induction of reactive oxygen species scavengers may also be involved in vanadate detoxification. Vanadate generates hydroxyl radicals ($OH^{\bullet}$) via a Fenton-like reaction [11], forms reactive oxygen species, including H_2O_2, in the presence of NADH [12,13], and causes lipid peroxidation in rats [14]. However, the ability of vanadate to induce oxidative stress in living organisms is controversial. In fact, having observed an antioxidant effect of vanadate on rat hearts, Matsubara and co-workers [15] have suggested that the oxidant properties of this metal ion can vary depending on both the cell line and the chemical form of the metal. Indeed, the effects exerted by vanadate on living systems still need to be fully elucidated. Vana-

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dium acts as a spermicide [16], mutagen and teratogen [17]. On the other hand, vanadium compounds can augment glucose transport, have insulin mimetic activities and be used in diabetes treatment [18,19]. Moreover, vanadium induces apoptosis in human cancer cells and may therefore be used as a new class of cytotoxic anticancer drugs [20].

In order to further understand the mechanisms implicated in vanadate toxicity in the yeast *H. polymorpha*, we have here compared the detoxification mechanisms activated in response to vanadate to those induced by copper, the effects of which in *S. cerevisiae* have been partly elucidated [21]. This approach is supported by the fact that even though specific molecular responses can be induced by toxic concentrations of certain heavy metals, common detoxification mechanisms can be triggered by a general heavy metal response. Furthermore, with the aim of further characterizing a controversial aspect of the cellular response to vanadate, we have also studied the oxidant properties of this anion in *H. polymorpha*.

2. Materials and methods

2.1. Strains, media and growth conditions

The yeast strains used were: NCYC1457 (also known as ATCC 34438), a wild-type strain of *H. polymorpha*, and the catalase deficient derivative MCT75 (*odc1 cat1*) [22]. The media used were GYNB (2% glucose, 0.7% Difco yeast nitrogen base without amino acids), VGYNB (GYNB plus 50 mM sodium orthovanadate), CGYNB (GYNB plus 0.4 mM copper sulphate), all at pH 5.8. Metal ions were added to the sterile medium from filter-sterilized stock solutions (sodium orthovanadate 500 mM and copper sulphate 50 mM). When necessary, uracil was added to the media at the final concentration of 19.9 mg l^{-1}. Unless otherwise stated, 1×10^6 cells ml^{-1} from overnight precultures in GYNB were used as inoculum. Cells were grown aerobically at 37°C in an orbital shaker (240 rpm). For growth rate and cell density evaluations, growth was followed by measuring the OD_{600} every 2 h (Cary 1E UV-VIS Varian spectrophotometer), using the supernatant as blank.

2.2. Enzymatic assays

Enzymatic activities were determined on crude extracts prepared as previously described by Mannazzu et al. [10]. Total superoxide dismutase (SOD) activity was assayed according to the method described by Beauchamp and Fridovich [23], in a final volume of 2.5 ml containing the crude extract in 50 mM phosphate buffer, pH 7.8, 60 μM nitroblue tetrazolium (NBT), 3 μM riboflavin and 10 mM methionine. One unit (U) of SOD activity in the crude extracts corresponds to a 50% inhibition in the reduction of NBT. Catalase (CAT) activity was assayed as described by Luck [24]. SOD and CAT activities are expressed as U (mg protein)$^{-1}$.

2.3. Induction of oxidative stress

Cells growing exponentially on GYNB were washed once in sterile distilled water, resuspended to a final concentration of 2×10^7 cells ml^{-1} in GYNB, and incubated for 1 h at 37°C in the presence of either 2.0 mM H_2O_2 or 0.2 mM plumbagin.

2.4. Preparation of protein samples for 2D electrophoresis

Between 0.5 and 1.0×10^9 cells were harvested, washed twice in sterile distilled water, and resuspended in 300 μl extraction buffer (200 mM Tris–HCl, pH 8.8, 20 mM $CaCl_2$, 100 μM $MgCl_2$, 0.2% SDS, 1% β-mercaptoethanol, 0.04% (wt/vol) RNAse, 1% phenylmethansulfonyl fluoride (PMSF) and 0.4% pepstatin A). About 0.7 vol glass beads (ϕ = 0.45 mm) was added, and the cells were vortexed for 30 s, boiled for 2 min, and cooled on ice for 5 min. After adding 4.0 μl PMSF and 2.5 μl pepstatin A, cell breaking was completed with seven cycles of 30 s vortexing interspersed with 30 s on ice. The supernatant was collected after 15 min centrifugation (10 000 × g at 4°C) and the crude extract was precipitated according to the method of Wessel and Flugge [25]. After resuspension in isoelectrofocusing sample buffer (9 M urea, 1% dithiothreitol, 2% 3–10 pharmalytes, 0.5% Triton X-100, 0.01% bromophenol blue), the samples were stored at −20°C.

Table 1
Changes in *H. polymorpha* protein expression levels during growth in the presence of vanadate (VGYNB) or copper (CGYNB)

Growth conditions	Number of proteins undergoing changes in their levels of induction						
	Newly synthesized	Up-regulated			Down-regulated		
		2–5-fold	5–10-fold	> 10-fold	2–5-fold	5–10-fold	> 10-fold
VGYNB	15	34	5	3	18	5	2
CGYNB	21	28	12	12	12	5	1

The protein profiles of cells growing on VGYNB or CGYNB were compared to those of cells growing on GYNB (control) by means of the Image 2D analyses software package (Pharmacia Biotech).

2.5. Two-dimensional gel electrophoresis

Proteins were resolved on two-dimensional gels by the method of O'Farrel [26], modified according to the manufacturer's instructions (Pharmacia Biotech). Fifteen μg proteins was separated on a precast Immobiline Dry Strip (Pharmacia Biotech) with a linear pH gradient (pH 3–10) for the first dimension, and on a precast ExcelGel SDS with an 8–18% (wt/vol) polyacrylamide gradient for the second dimension. Gels were stained with a Pharmacia silver staining kit and analyzed with the image analysis software Image master 2D (Pharmacia Biotech).

2.6. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined by a modification of the method previously described by Howlett et al. [27]. Briefly, cells growing exponentially on GYNB were harvested, washed twice in ice-cold sterilized distilled water, resuspended in OG buffer (10 mM 2-[*N*-morpholino]ethanesulphonic acid (MES), pH 5.5, 1% glucose), VG buffer (OG plus 50 mM sodium orthovanadate) or CG buffer (OG plus 0.4 mM copper sulphate) to a final concentration of 1×10^8 cells ml^{-1}, and incubated in an orbital shaker at 37°C. TBARS determination was performed on 1×10^8 cells ml^{-1} after 30, 60, 120, 240, and 480 min from the resuspension. Absorbance of the samples at 535 nm was measured against a reference solution containing 1 ml of TBA reagent and 500 μl sterilized distilled water using a Cary 1E UV-VIS Varian spectrophotometer. The concentration of TBARS in the samples was calculated by reference to a standard curve prepared by using 1,1,3,3,-tetramethoxypropane.

3. Results

3.1. The molecular responses to vanadate, copper and oxidative stress show common determinants

Two-dimensional SDS–polyacrylamide gel electrophoresis was utilized to analyze the changes in protein expression caused by the presence of vanadate or copper in the growth medium. Computerized analysis of the gels revealed that both metal ions induce marked alterations in the protein profiles in comparison to the control (Table 1). Interestingly, at least 38 proteins showed similar alterations in expression levels in both vanadate- and copper-containing media, indicating the existence of a significant overlap in the cellular responses to both metals (Fig. 1).

It has previously been shown that genes involved in oxidative stress protection are regulated by metal ion responsive transcription factors [28], and that copper detoxification is mediated in part by the activation of an antioxidant response [6,7]. Therefore, the protein expression patterns of cells growing on VGYNB and CGYNB were

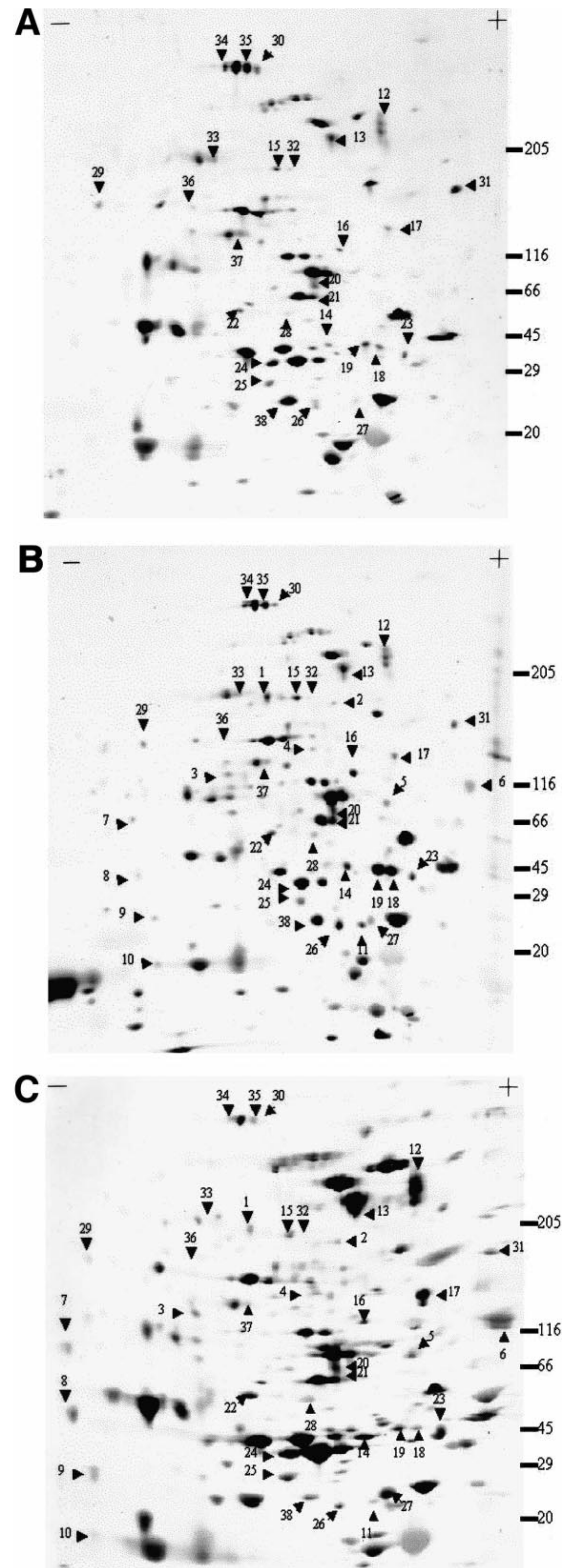

Fig. 1. 2D electrophoresis profiles of cells growing exponentially on GYNB (A), VGYNB (B) and CGYNB (C). Numbered arrows refer to newly synthesized (1–11), up-regulated (12–28) and down-regulated (29–38) proteins. Molecular mass standards are indicated on the right of the gel in kDa.

Table 2
Effects exerted by vanadate and copper on antioxidant enzyme levels in NCYC1457 cells

Growth conditions	Enzymatic activities U (mg protein)$^{-1}$	
	SOD	CAT
GYNB	27.23 ± 1.9	47.1 ± 2.1
VGYNB	73.97 ± 5.2[a]	441 ± 34.2[b]
CGYNB	60.09 ± 22.6[a]	127.8 ± 15.3[a]

Data are means ± S.D. of three independent experiments.
[a]Significantly different from value for GYNB ($P < 0.05$).
[b]Significantly different from values for GYNB and CGYNB ($P < 0.001$).

compared to those of cells treated with sublethal doses of H_2O_2 and plumbagin, sources of peroxide and superoxide anions respectively. Intriguingly, significant overlaps in the protein expression patterns were observed. At least 74% of the proteins which were newly synthesized or expressed at higher or lower levels in both VGYNB and CGYNB showed similar alterations in expression levels in cells treated with the two pro-oxidants utilized (Fig. 2), thus suggesting that heavy metal detoxification may be mediated, at least in part, by the oxidative stress response in *H. polymorpha*.

3.2. Vanadate and copper enhance antioxidant enzyme activities

Since yeast cells counterbalance the increased production of reacting oxygen species by also enhancing the activities of antioxidant enzymes, we measured the levels of SOD and CAT activities in NCYC1457 cells during exponential growth on GYNB (control), VGYNB and CGYNB. The presence of either metal ion in the growth medium caused a significant increment in the activity of the two enzymes (Table 2). Interestingly, while vanadate and copper induced comparable increments in the levels of SOD activity (1.7-fold on VGYNB and 1.2-fold on CGYNB), dramatic differences were observed in the effects of the two metal ions on CAT activity, which showed an 8.4-fold increment on VGYNB and a 1.7-fold increment on CGYNB. Notably, the CAT activity increment on VGYNB was comparable to that induced by treatment of NCYC1457 cells with sublethal doses of H_2O_2 (9.8-fold; data not shown).

To investigate the role of CAT activity in vanadate tolerance the wild-type strain, NCYC1457, and its CAT deficient derivative, MCT75, were inoculated into vanadate-containing medium. Intriguingly, MCT75 cells were able to grow on VGYNB, indicating that CAT activity is not necessary for the natural vanadate tolerance of *H. polymorpha*. However, MCT75 cell growth rates were lower than those of NCYC1457 in both GYNB and VGYNB (Table 3), and the CAT deficient mutant reached a cell density lower than that of the parental strain under both growth conditions (data not shown).

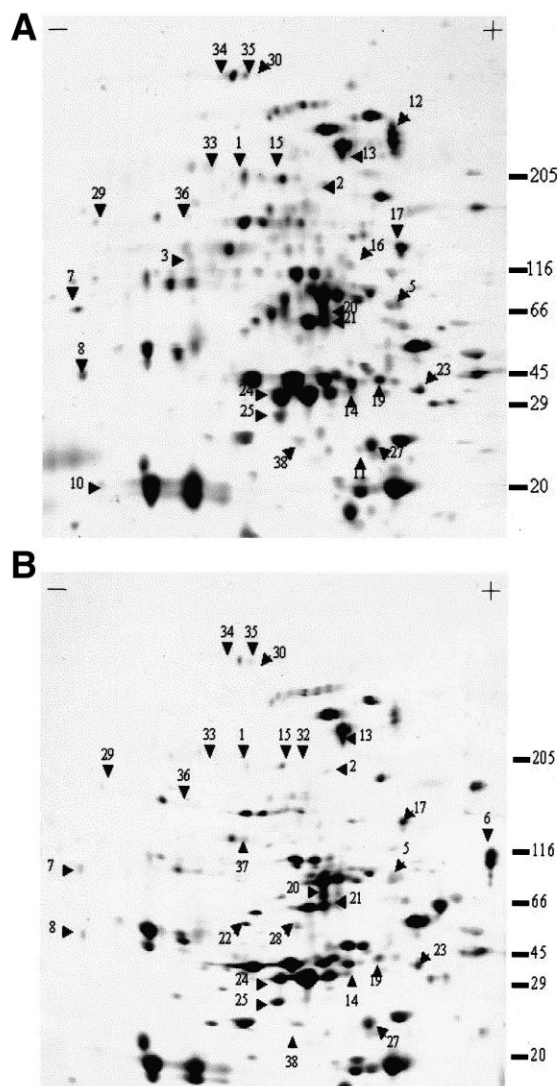

Fig. 2. 2D electrophoresis profiles of cells subjected to sublethal treatments with H_2O_2 (A) and plumbagin (B). Numbered arrows indicate all the proteins with altered expression levels in common with VGYNB and CGYNB (see Fig. 1). Molecular mass standards are indicated on the right of the gel in kDa.

Table 3
Generation times in the catalase deficient strain MCT75 and the parental strain NCYC1457

Growth conditions	Generation time (h)	
	NCYC1457	MCT75
GYNB	1.15 ± 0.23	2.14 ± 0.09[a]
VGYNB	5.60 ± 0.44	6.43 ± 0.41[a]

Data are means ± S.D. of three independent experiments.
[a]Significantly different from value for NCYC1457 ($P < 0.05$).

3.3. Vanadate and copper exert different effects on cellular membranes

Lipid peroxidation of membranes can be followed by measuring the intracellular levels of malondialdehyde (MDA), which is mainly produced by decomposition of

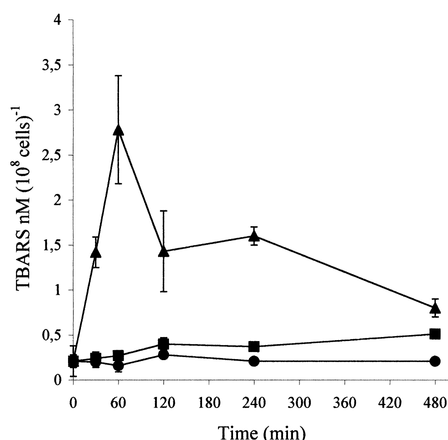

Fig. 3. TBARS production in NCYC1457 cells under control conditions (OG, ●) and after exposure to vanadate (VG, ■) and copper (CG, ▲). Error bars represent standard deviations ($n = 3$).

lipid hydroperoxides caused by reactive oxygen species [27]. To assess whether copper and vanadium can cause peroxidation of cellular membranes, the amount of MDA produced by *H. polymorpha* NCYC1457 cells in the presence of either metal was measured by means of the TBARS method. While copper caused an increment in MDA production, with a peak at 60 min from the addition of the metal, vanadate did not induce any change in the levels of MDA after up to 8 h of contact with the cells (Fig. 3).

4. Discussion

In order to gain further insights into the understanding of vanadate toxicity on living organisms, and assuming that the cellular response to different heavy metals can be mediated, at least in part, by common determinants, we have compared the modifications in protein expression induced by vanadate and copper in the yeast *H. polymorpha*. The presence of either metal induces evident changes in the protein expression patterns. Although some of these changes may be a sign of a metal-induced disturbance of the cellular metabolism, they can also be caused by the overexpression of molecules involved in the detoxification processes, and the down-regulation of unnecessary proteins, with the aim of lowering energy consumption, critical under stressful conditions [29]. Moreover, as heavy metal tolerance can be mediated by the exclusion of the metal ions from the cellular compartment, the observed decreases in the level of expression of certain proteins could also be due to the repression of the transcription of genes coding for metal transporters, as seen in *S. cerevisiae* under conditions of high copper concentrations [30].

The changes in the protein expression patterns induced by vanadate and copper show marked similarities, with at least 38 proteins being expressed at higher or lower levels in the presence of either metal, thus suggesting the existence of common determinants in the detoxification of these two metals. While these changes in protein expression are not always of the same magnitude, as might be expected from the full reactivity profiles of these two heavy metals, the fact that at least 28 of these proteins also behave in a similar way in response to sublethal concentrations of H_2O_2 or plumbagin indicates that the induction of an antioxidant response is one of such common determinants. Indeed, the significant increases in the activities of the antioxidant enzymes SOD and CAT caused by vanadate and copper confirmed that both metal ions induce an antioxidant response in *H. polymorpha*.

Interestingly, vanadate exerts a more dramatic effect on CAT levels than copper. Catalase, localized in the peroxisomes in *H. polymorpha* [22], is characterized by high H_2O_2 reduction rates and by a high efficiency in the elimination of the hydrogen peroxide produced by the peroxisomal oxidases. However, this enzyme shows an affinity for H_2O_2 which is at least three orders of magnitude lower than the affinity of cytochrome *c* peroxidase (CCP) for the same substrate [31]. Taking this into account, and considering that CAT is not essential for growth on VGYNB, the observed vanadate-induced increment in CAT activity is somewhat surprising. A possible explanation for this can be found in the evaluation of the consumption of biologically useful energy connected with CCP activity. In fact, the action of CCP circumvents ATP generation at the third proton translocating loop (site III) of the electron transport chain, and results in a decrease in biomass production [31]. ATP is strongly required for the repair and elimination of damaged molecules during oxidative stress [29]. Grant and co-workers have hypothesized that the repair of damaged proteins, the detoxification of lipid peroxidation products, and the active transport to the vacuole of oxidized and aberrant proteins for breakdown reactions may require energy [29]. Moreover, it has been shown that the mitochondrial CCP can substitute for the peroxisomal catalase in *H. polymorpha* catalase-defective mutants [32]. Therefore, it is plausible that cells growing in unfavorable conditions can transfer the detoxification of H_2O_2 from CCP, highly specialized but energy consuming, to the less costly catalase. Interestingly, MCT75 cells, while being able to grow in the presence of vanadate, show lower growth rate and biomass production, on both GYNB and VGYNB, when compared to the parental strain. These results suggest that, even though not essential for growth on vanadate-containing medium, CAT can represent a useful auxiliary route for the elimination of H_2O_2 which is switched on when cells are under adverse conditions. Surprisingly, vanadate seems to be more effective than copper in the activation of this route. We have also shown that while copper causes lipid peroxidation in *H. polymorpha*, as has also been observed in *S. cerevisiae* [27], vanadate does not, in antithesis to what has been reported by Harvey and Klaasen [14] in a different biological system. Therefore, our results indicate that, besides

the existence of significant overlaps in the cellular responses to vanadate and copper, additional protective circuits can be activated against vanadate-induced oxidative insults by *H. polymorpha*.

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