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Recovery from Vanadium Involves the Elimination of Cellular Structures in the Yeast *Hansenula polymorpha*

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Summary

The mechanisms by which living organisms can protect themselves from vanadium toxicity are not completely understood. This is partly due to the multiplicity of possible targets for the metal which interferes with the phosphorylation state of enzymes and regulatory molecules. In vanadate-resistant mutants of Neurospora crassa and Candida albicans resistance to vanadium is due to the exclusion of the metal from the intracellular compartment through the inactivation of the phosphate transport system. Saccharomyces cerevisiae vanadate-resistant mutants show glycosylation defects and alterations in protein phosphorylation and growth control which suggest that resistance could be due to the alteration of sensitive target(s) in the secretory pathway and/or in the phosphorylation system. The vanadate-tolerant yeast Hansenula polymorpha greatly modifies its ultrastructural aspect when grown in the presence of the metal, showing an increase in cell vacuolation and in the number of cytoplasmic vesicles, and a thickening of the cell wall. At the same time, the external invertase activity is partially inhibited and the carboxypeptidase Y activity is enhanced. In order to understand better the meaning of these observed modifications we followed their evolution during return to vanadate-free conditions. Our results show that recovery from vanadium is a complex phenomenon involving important ultrastructural rearrangements, which could be achieved through the activation of an autophagic process.

Keywords: vanadium, *H. polymorpha*, metal tolerance, ultrastrucure

Introduction

The mechanisms by which living organisms can protect themselves from vanadium toxicity are not completely understood. However, the addition of vanadate both *in vivo* and *in vitro* indicates that one of its main metabolic targets is the phosphorylation state of enzymes and other regulatory molecules, such as the inhibition of phosphofructokinase and adenylate kinase (*1*), acid and alkaline phosphatases (*2*), P-type ATPases (*3*) and RNAses (*4*). Furthermore, *in vivo* addition of vanadate has also been shown to increment protein phosphorylation (*5,6*), chloride transport (*7*), cAMP levels (*8*) and DNA synthesis (*9*). Although very little is known about these latter stimulatory effects of vanadate addition (involving the consequent reduction of vanadate to vanadyl *in vivo*), many of the effects of vanadate have been attributed to its structural and metabolic similarity to phosphate. In fact, it has been demonstrated that vanadate can enter the cell *via* the phosphate transport system in erythrocytes (*3*), *Neurospora crassa* (*10*) and *Saccharomyces cerevisiae* (*11*). Symptomatic of this mode of entry of vanadate into cells, in vanadate-resistant mutants of *Neurospora crassa* and *Candida albicans* this resistance is due to the exclusion of the metal from the intracellular compartment through the inactivation of the phosphate transport system (*12,13*).

However, in the case of *Sacch. cerevisiae*, vanadate has been shown to accumulate intracellularly, hence leading to the formation of toxic high molecular mass com-

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pounds, and a cessation of growth (*11*). Furthermore, these vanadate-resistant mutants show glycosylation defects and alterations in protein phosphorylation and growth control (*14–16*), which suggests that this resistance is due to the alteration of intracellular targets in the secretory pathway and/or the phosphorylation system. In further support of this, the proteins that are involved in the organisation and functioning of the Golgi apparatus and the secretory pathway are coded for by genes implicated in vanadate resistance that have been isolated (*14,16*). Hence, and as indicated by Kanik-Ennulat *et al.* (*16*), the decreased toxicity of vanadate and the general plasma membrane and cell wall defects observed in *Sacch. cerevisiae* vanadate-resistant mutants could be due to either a proliferation of the Golgi apparatus or a modification of vesicle or membrane targeting at the level of the Golgi.

The vanadate-tolerant yeast *H. polymorpha* undergoes ultrastructural modifications during growth on vanadate-containing medium (*17*). In this report we present data on the evolution of these modifications and on the activities of secretory and proteolytic marker enzymes in *H. polymorpha* during a return to vanadate-free conditions.

Methods

Growth conditions

The *H. polymorpha* strain NCYC 495 was used. Media used were GYNB (2% glucose, 0.7% Difco Yeast Nitrogen Base), VGYNB (GYNB plus 50 mM sodium orthovanadate). Sodium orthovanadate (Sigma) was added to the sterile medium from a filter-sterilized stock solution of 500 mM, pH = 5.8. Cells were grown at 37 °C in an orbital shaker (240 min^{-1}).

Recovery from vanadate

Cells grown to mid-exponential phase on VGYNB or GYNB were harvested by centrifugation, washed two times in H_2O and reinoculated in GYNB to a final concentration of $2.5 \cdot 10^7$ cell mL^{-1}. Samples for cell counting and ultrastructural and enzymatic analyses were taken immediately after the transfer to GYNB (time zero) and after further 30 and 240 min^{-1}.

Ultrastructural analyses

Ultrathin sections (50 nm) were prepared and stained for TEM as previously reported (*17*), with vesicle number and size being determined on photographic prints (final magnification: 40,000 ×). Vesicles were scored as electron dense disks or rings of 35–45 nm. Vesicle counts were normalized for cell volume, which was calculated by measuring the area of the cell section (measured from the plasma membrane), and using a section thickness of 50 nm.

Enzymatic assays

External invertase (ExI) activity was assayed on whole cells as described by Goldstein and Lampen (*18*), modified as follows: the enzymatic and colorimetric reactions were carried out at 37 °C for 10 and 20 min, respectively. The enzymatic reaction was stopped by the addition of 0.3 mL 0.2 M K_2HPO_4, pH = 10; the colorimetric reaction was stopped by the addition of 2 mL 6M HCl. ExI activity was calculated in mmoles of glucose released per min, and related to the sample dry mass. Carboxypeptidase Y (CPY) activity was assayed on crude extracts according to Jones *et al.* (*19*), and calculated in mmoles of *p*-nitroaniline produced per min per mg of protein. These enzyme activities were then related to the control (no vanadate treatment) activities, and expressed as percentage.

Preparation of crude extracts

About $0.5–1 \cdot 10^9$ cells were harvested by centrifugation, washed twice in H_2O and resuspended in 0.2 mL 0.1 M tris-HCl, pH = 7.6. About 0.7 volume of glass beads (2r = 45 mm, Sigma) was added, and the cells were broken by eight cycles of 30 s vortexing interspersed with 30 s in ice. The supernatant was collected by a 10 min centrifugation in a microcentrifuge. The protein concentration of the extracts was determined according to Bradford (*20*), using BSA as standard.

Results

We have previously reported that *H. polymorpha* cells grown on vanadate (50 mM) show a number of ultrastructural modifications, including a thickening of the cell wall and an increase in cell vacuolation and in the number of cytoplasmic vesicles (*17*). Upon return of vanadate-treated cells to control conditions (GNYB; see Methods), a delay in the growth recovery of some 30 to 60 min was observed (data not shown) before these cells were able to resume the control growth rate. We have therefore related ultrastructural and enzymatic activity alterations in cells immediately after the return to GYNB (zero time) to those after 30 and 240 min of recovery from vanadate (Fig. 1). As illustrated in Fig. 1A, zero-time cells demonstrated an ultrastructure typical of *H. polymorpha* under vanadate growth conditions, as described above. Furthermore, during the growth recovery delay (30 min post-vanadate) these cells not only showed an increase in the amount of electrondense material present in the vacuoles (Fig. 1B), but also a further enhancement of the CPY activity (Fig. 2; 30 min), indicative of increased vacuolar proteolytic activity. After a further recovery period of 210 min (Figs. 1C and 2; 240 min), both cellular ultrastructure and CPY activity had returned to those typical of normal GYNB cell growth, with the majority of cells showing a decrease in cell wall thickness and vacuole size.

Another aspect of these vanadate-treated cells is an increase in the number of 40 nm vesicles that are visible in their cytoplasm (Fig. 3A; zero time) that is associated with a decrease in the ExI activity (Fig. 3B; zero time), indicative of a perturbation of a late stage in the secretory pathway. As seen with the CPY activity, during the growth recovery delay the number of vesicles showed a further increase (Fig. 3A; 30 min), at which time the ExI activity remained at the vanadate-treated level (Fig. 3B; 30 min). However, after the full recovery period, both vesicle numbers (Fig. 3A; 240 min) and ExI activity (Fig. 3B; 240 min) were at similar levels to GYNB-grown cells.

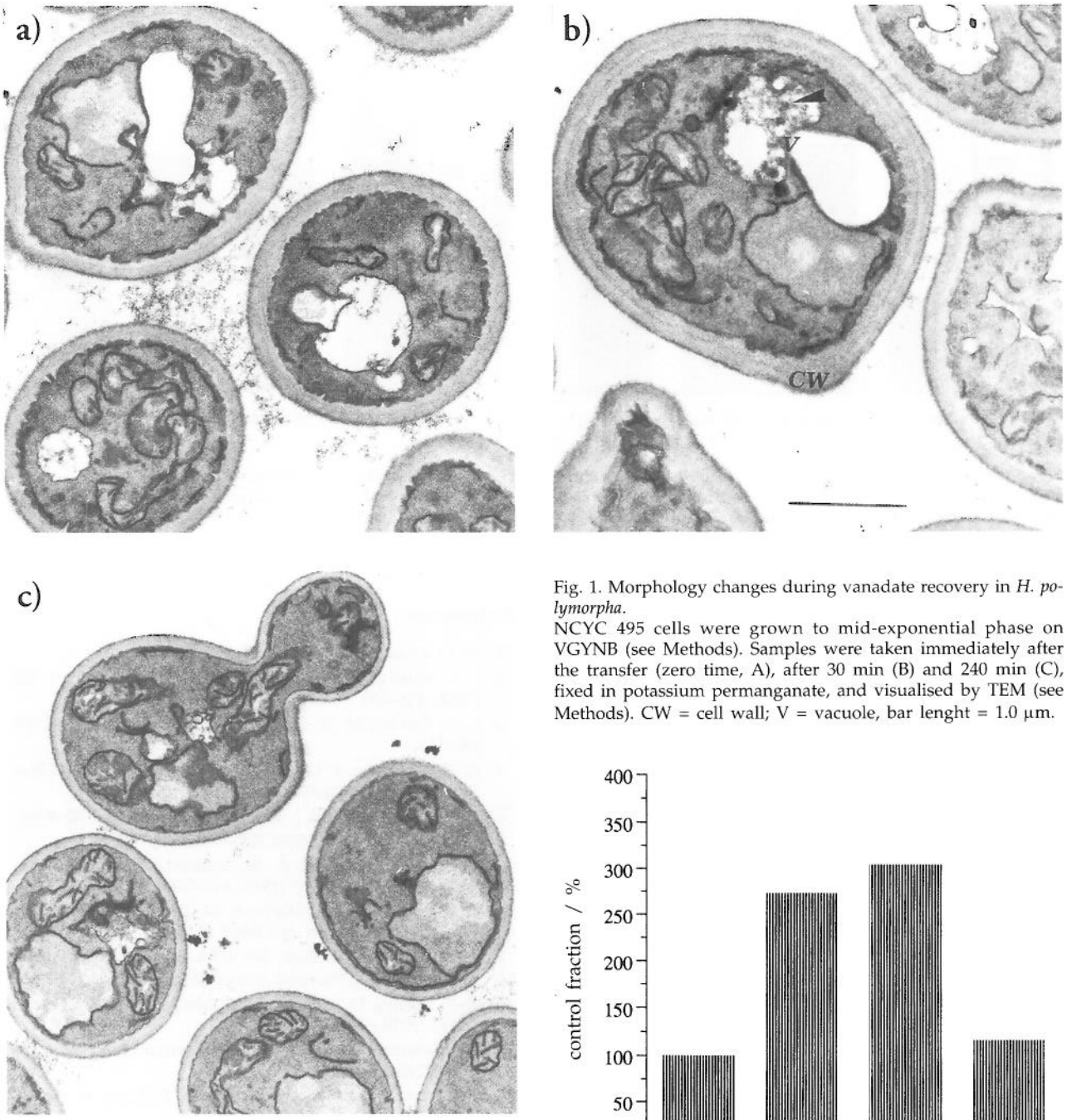

Fig. 1. Morphology changes during vanadate recovery in *H. polymorpha*.
NCYC 495 cells were grown to mid-exponential phase on VGYNB (see Methods). Samples were taken immediately after the transfer (zero time, A), after 30 min (B) and 240 min (C), fixed in potassium permanganate, and visualised by TEM (see Methods). CW = cell wall; V = vacuole, bar lenght = 1.0 μm.

Fig. 2. Vacuolar proteolytic activity changes during vanadate recovery in *H. polymorpha*.
Cell samples were taken before vanadate treatment (control) and during vanadate recovery at the times indicated. CPY activities were assayed as detailed in Methods. Data are from a single experiment and are representative of three independent experiments. Vanadate recovery sample activities were expressed as a percentage of the control activity of 1.12 ± 0.05 U mg^{-1}.

Discussion

H. polymorpha cells growing in vanadate-containing medium show a thickening of the cell wall and an increase in cell vacuolation and in the number of small cytoplasmic vesicles (*17*). Furthermore, these vanadate-growth conditions also lead to increased CPY activity and decreased ExI activity (Mannazzu and Guerra, unpublished results). Taken together, these cellular changes indicate that in the presence of vanadate there is an inhibition of processes involved in secretion and an enhancement of vacuolar activity in *H. polymorpha*.

In this report we demonstrate initially that these modifications induced by vanadate in *H. polymorpha* are not only reversible, but that they also undergo further modifications *prior* to the return to normal (vanadate-free) growth conditions. During this early (30 to 60 min) vanadate-recovery period, this further enhancement of

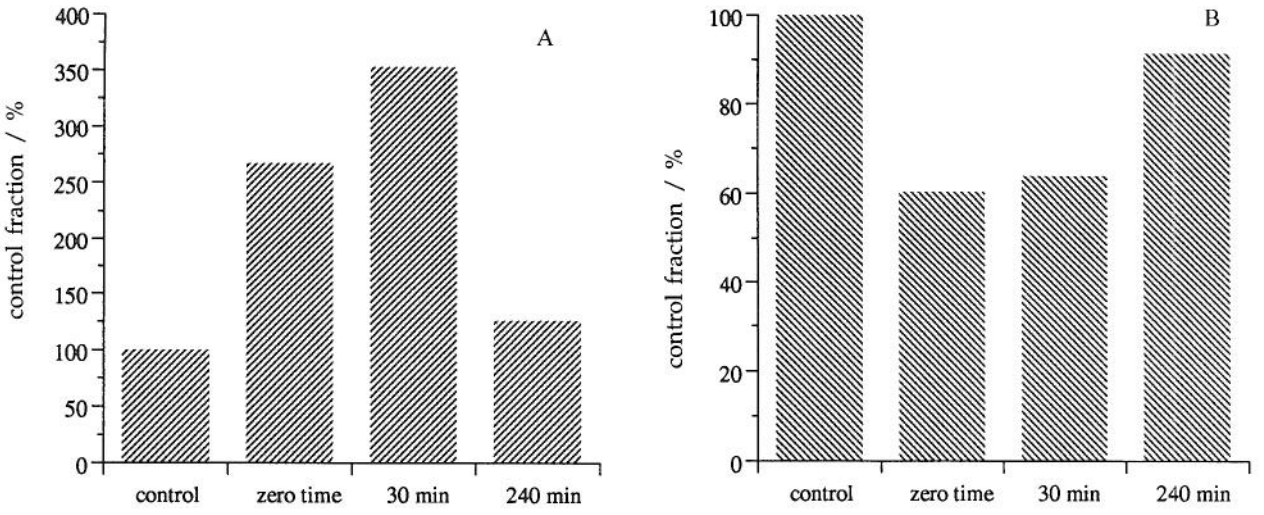

Fig. 3. Vesicle number (A) and secretory activity (B) changes during vanadate recovery in *H. polymorpha*.
Cell samples were taken before vanadate treatment (control) and during vanadate recovery at the times indicated. Vesicle numbers and ExI activities were determined as detailed in Methods. Data for vesicle numbers are from a sample size of 50 cells, and data for ExI activity are from a single experiment and are representative of three independent experiments. Vanadate recovery samples were expressed as a percentage of the control values of 54.6 ± 4.3 for vesicle numbers (A), and 0.116 ± 0.001 U mg^{-1} dry mass for ExI activity (B).

vacuolar proteolytic activity, as demonstrated by the high levels of CPY activity, is concomitant with an increase in the amount of electron-dense material observed inside the vacuoles. As CPY is known to be involved in the degradation of proteins and peptides following adaptation to limiting nutrition conditions (*21*), this enhanced CPY activity that appears necessary before the resumption of normal growth suggests the activation of an autophagic mechanism during this recovery period. Furthermore, the ability of *H. polymorpha* to recover from vanadate-induced toxicity, while at the same time demonstrating disruptions in secretory vesicle processing (as seen by vesicle accumulation and the decrease in ExI activity), is supported by the different routes for invertase and CPY in the later stages of the secretory pathway; invertase is packaged in secretory vesicles and delivered to the plasma membrane, while CPY is delivered to the vacuole by a different route (*21*).

The ability of *H. polymorpha* to resist vanadate toxicity could be associated with the presence of aberrant cellular structures (*17*), as they have also been observed in vanadate-resistant mutants of *Sacch. cerevisiae* (*16*). Zoroddu *et al.* (*22*) have also indicated sites of vanadate/vanadyl accumulation through the formation of complexes with cell wall polymers in *H. polymorpha*. At this stage though, further studies are needed to determine whether such structures are indeed an integral part of vanadate resistance, or just a consequence of vanadate toxicity. However, in the present report we demonstrate that in order to resume normal cell growth upon the return to vanadate-free conditions, there is a need not only for the elimination of vanadium-containing compounds, but also for the elimination of these vanadate-induced aberrant cellular structures through a potentially autophagic process.

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Oporavak od trovanja vanadijem uključuje razgradnju staničnih struktura kvasca *Hansenula polymorpha*

Sažetak

Još nisu do kraja ispitani mehanizmi kojim se živi organizmi mogu zaštititi od toksičnog djelovanja vanadija. Uzrok je tome mnogostrukost mogućih mjesta na koja se metal može vezati, što utječe na stanje fosforiliranosti enzima i regulatorskih molekula. Mutanti Neurospora crassa i Candida albicans svoju otpornost na vanadate postižu isključivanjem metala iz intracelularnih odjeljaka inaktivacijom sustava za prijenos fosfata. Na vanadij otporni mutanti Saccharomyces cerevisiae pokazuju nedostatke u glikozilaciji, promjene u fosforilaciji proteina i kontroli rasta, što upućuje na to da uzrok otpornosti može biti promjena na bitnom mjestu sekrecijskog puta i/ili u sustavu fosforilacije. Vanadat tolerantni kvasac Hansenula polymorpha, u prisutnosti metala, znatno mijenja svoju ultrastrukturu, povećavajući broj vakuola i citoplazmatskih vezikula u stanici zadebljavajući stanični zid. Istodobno, djelomično je inhibirana aktivnost eksterne invertaze, a pojačana aktivnost karboksipeptidaze Y. Da bi se bolje objasnile opažene promjene, praćen je njihov razvoj tijekom povrata u stanje bez vanadata. Dobiveni rezultati pokazuju da je oporavak od vanadija složen fenomen koji obuhvaća znatne ultrastrukturne preinake što su vjerojatno posljedica aktiviranja procesa autofagije.