7-16-2009

An Analysis of Glycolytic Enzymes in the Cellular Response to Metal Toxicity

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AN ANALYSIS OF GLYCOLYTIC ENZYMES IN THE CELLULAR RESPONSE TO METAL TOXICITY

by

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Under the direction of Dr. John E. Houghton

ABSTRACT

Metal toxicity is implicated in neurotoxicity, nephrotoxicity, aging and cancer. Protein oxidation resulting from oxidative stress is now known to be involved in metal toxicity. However, proteomic responses to metal induced oxidative stress have not been characterized. By using the yeast as a model, we characterized these changes occurring in response to sub-lethal doses of metals. Several proteins involved in protein synthesis, ribosome assembly decreased while antioxidant defenses, proteins involved in sulfur metabolism, and glutathione synthesis and ubiquitin increased following metal exposure. We also show that metals induced temporal and targeted protein oxidation independent of protein abundance. Among the targets were glycolytic enzymes and heat-shock proteins. As a consequence, glycolytic enzyme activities decreased whereas the levels and activities of the enzymes of the alternative pathway for glucose metabolism, pentose phosphate pathway (PPP) increased. True to prediction, we also found increased flow through the PPP as measured by elevated levels of NADPH and glutathione. NADPH and glutathione are crucial

for maintaining the redox balance in the cell. Thus, rerouting of glucose metabolism into PPP is considered to be beneficial to the organism. Among the oxidation targets is a glycolytic protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that is required for apoptosis in neuronal cells. We show that not only is GAPDH required for metal induced apoptosis in yeast but also the levels of GAPDH transcript and protein increase in the cytosol and the nucleus in an isoform specific fashion. Such changes strongly implicate the role of GAPDH in yeast apoptosis. This work provides evidence for the involvement of targeted protein oxidation in metal toxicity, shows the overlaps and differences in the mechanism of copper and cadmium toxicity, allows comprehension of how metabolic processes respond to metal stress and explores the potential of GAPDH as a sensor of oxidative stress and mediator for apoptosis.

INDEX WORDS: Metal Toxicity, Oxidation, Oxidative stress, GAPDH, Yeast, Glycolytic flux, PPP

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2008

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August 2008

DEDICATION

This is to my husband, Manoj Muthukumar, for all his support, understanding, and endless patience and for believing in me more than I do in myself.

To my son, Maadhav Muthukumar for all the joy he has brought me, which has sustained me through the completion of this major effort.

To my parents and my brother for their continued support and encouragement.

To all those who struggle, falter and persevere to achieve something.

ACKNOWLEDGEMENT

I thank my advisor, Dr. John E. Houghton for his helpful comments, valuable advice and support during this research undertaking. I am much grateful to Dr. Houghton for being patient with me and never letting me give up on research.

I also thank my committee members – Dr. Simon Avery, Dr. Barbara Baumstark and Dr. Zehava Eichenbaum for their guidance and support. My thanks to my peers and colleagues – Amrita Nargund, Liangyong Jiang, Divya Rao, Suchita Sood, Bishu Sapkota, Prajakta Pradhan, Ling Wei, Rollin Dennard and Debby Walthall for their friendship, technical help.

I would also thank the Molecular Basis of Disease Fellowship at Georgia State University for supporting me.

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An Analysis of Glycolytic Enzymes in the Cellular Response to Metal Toxicity

General Introduction

Why study metal toxicity?

Metal exposure is implicated in nephrotoxicity (Fukumoto *et al*., 2001), neurotoxicity, genotoxicity (Bagchi et al., 2000), carcinogenicity (Degraeve, 1981; Desoize, 2003; Waalkes, 2003) and aging (Oliver *et al*., 1987; Sohal and Dubey, 1994). Moreover, the importance of maintaining copper (Cu) homeostasis is reflected in Menke's disease (copper deficiency), Wilson's disease (copper excess) (Kletzien et al., 1994) and autism (Kern *et al*., 2007). Metal-catalyzed oxidative stress and oxidation of proteins are implicated in the pathogenesis of a number of neuro-degenerative disorders such as Alzheimer's disease (Becaria et al., 2003; Bush, 2003), Parkinson's disease (Jenner, 2003), amyotrophic lateral sclerosis (Ciriolo et al., 1994) and autism (Kern et al., 2007) as well as in aging (Stadtman, 2004). While the physiological and macroscopical effects of metal exposure have been studied extensively, the molecular mechanisms of metal toxicity remain less well chracterized.

Yeast as a model system to study metal toxicity

To study metal toxicity, yeasts, particularly *Saccharomyces cerevisiae* are excellent models for a number of reasons. Cellular function is highly conserved between yeasts and mammalian cells. With particular reference to metal toxicity, effects of and defenses against

oxidative stress are common to yeasts and higher eukaryotes. There is also information available regarding metal-homeostasis and yeast-metal interactions. Moreover, *S. cerevisiae* can be easily manipulated genetically, which is more difficult to do in other model systems of toxicity such as filamentous fungi and higher eukaryotes. Also, the genome and proteome of *S. cerevisiae* have been characterized in recent years. All these reasons make *S. cerevisiae* an excellent eukaryotic model to understand metal toxicity (Avery, 2001).

Metal toxicity and ROS

Metals are known to generate elevated levels of reactive oxygen species (ROS). ROS is a collective term for superoxides (O_2^-), peroxides (H_2O_2) and the highly reactive hydroxyl radicals (OH^o) (Halliwell and Gutteridge, 1999). Metals differ in their method of ROS production. On the one hand, redox-active metals such as iron (Fe), chromium (Cr) and copper (Cu) are capable of catalyzing the Fenton and Harber-Weiss reaction, which can generate the highly potent hydroxyl radical from the byproducts of aerobic respiration, O_2^- and H_2O_2 as follows.

$$2O_2^{\circ -} + 2H+ \longrightarrow H_2O_2 + O_2 \qquad \text{(Dismutation)}$$

$$O_2^{\circ -} + M^{n+} \longrightarrow O_2 + M^{(n-1)+}$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + OH^{\circ} + OH^-$$

On the other hand, redox-inactive metals such as cadmium (Cd), lead (Pb) and mercury (Hg) are incapable of taking part in the above reaction series of reaction. However, they can still generate ROS either by displacing redox-active metals from active sites of enzymes (Wei et al., 2001), by disabling antioxidant defenses such as glutathione (Stohs and Bagchi, 1995), or by perturbing the mitochondria (Pacheco *et al*., 2007), which are the major sites of ROS production in the cell.

All cells undergoing aerobic metabolism have to contend with ROS and, therefore, are equipped with anti-oxidant defenses. There are the enzymatic defenses such as superoxide dismutase, catalase, glutathione peroxidase and the non-enzymatic defenses such as glutathione; thioredoxin etc. which, together, either quench the ROS or alleviate ROS induced damage. However, when the levels of ROS overwhelm the antioxidant defenses, as can occur with metal exposure, a condition called oxidative stress results. Although it is universally agreed that metals induce oxidative stress, whether or not oxidative stress is the mechanism of metal toxicity is as yet not completely understood.

Intracellular targets of ROS

ROS molecules, the mediators of oxidative stress, are extremely potent and harmful and can react with, and damage, cellular macromolecules such as DNA, lipids, proteins and carbohydrates as shown in Figure 1 and Figure 2. Oxidative damage to DNA occurs in response to metal exposure and results in base modifications, depurination, DNA and protein cross-linking and strand-scission. Depurination and strand-scission are known to induce mutations in the DNA, manifesting as genotoxicity and carcinogenicity. A major product of DNA oxidation is 8-oxoguanine (Cadet *et al*., 2003). However, deletion of *OGG1* – the only yeast glycosylase specific for repair of 8-oxoguanine (Boiteux et al., 2002; Leipold et al., 2003) had no discernable effect on sensitivity to Cu, Cd and Cr (Sumner *et al*., 2005). Also, it has been recently shown that DNA damage resulting from Cd exposure is actually secondary, arising as a result of damage to the DNA mismatch repair proteins (Jin et al., 2003).

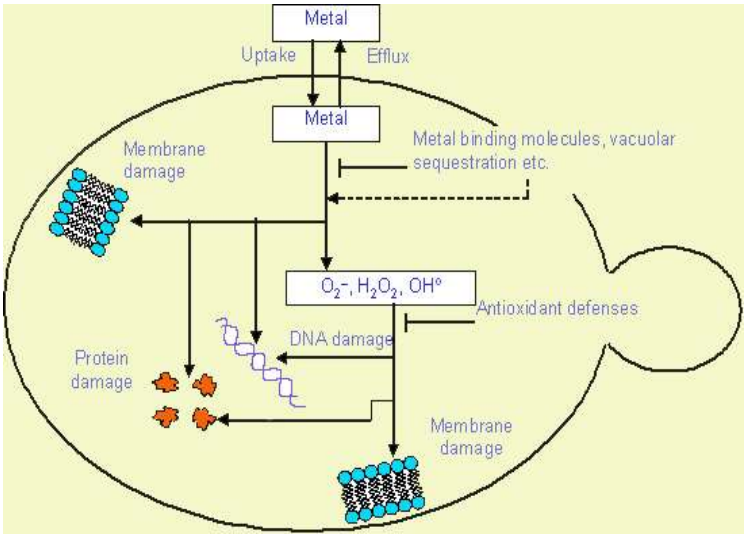

Figure 1. Metal toxicity as depicted in a yeast cell

Metal-induced ROS generation and its effects on cellular macromolecules are depicted (Avery, 2001).

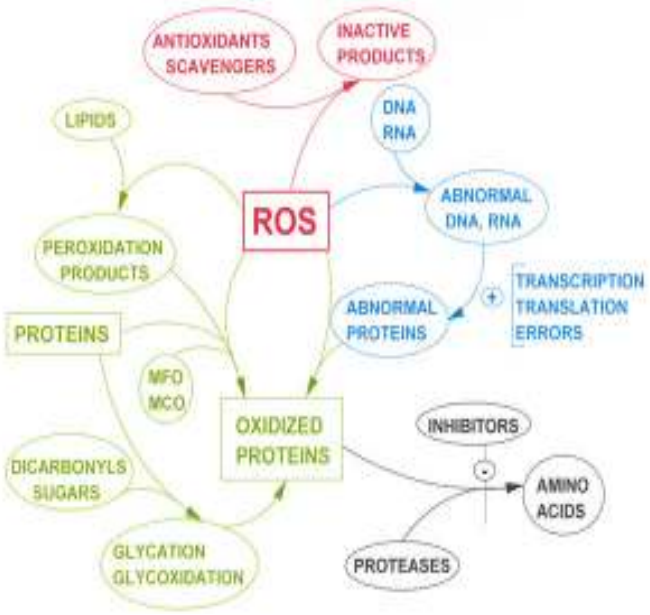

Figure 2. Damage induced by ROS on cellular macromolecules

Proteins can be oxidized by ROS themselves, by lipid peroxidation products and abnormal proteins synthesized from damaged nucleic acids. Oxidized proteins are earmarked for proteolysis.

Therefore, it seems likely that although DNA damage does occur in response to metal exposure, it is coincidental, secondary and potentially not responsible for metal toxicity.

Lipids are another target of oxidative stress. Oxidative damage to lipids (lipid peroxidation) results in the conversion of unsaturated membrane lipids to lipid hydroperoxides (Fernandes et al., 2002; Howlett and Avery, 1997b), which increase the fluidity of the cell membrane. Extensive lipid peroxidation, as with metal stress, can result in increased cell fluidity and permeability (Howlett and Avery, 1997b), eventually leading to loss of cell integrity and ultimately cell death. Moroever, cadmium toxicity is dependent on the fatty acid composition of the cell (Avery et al., 1996; Howlett and Avery, 1997a), suggesting that lipid damage is involved in Cd toxicity. Lipid peroxidation is repaired by phospholipid hydroperoxide glutathione peroxidases (PHGPxs), which reverse the oxidative damage to biological membranes. While deletion of PHGPxs had no effect on Cr toxicity (Sumner *et al*., 2005), it was deleterious to Cd exposed cells (Shanmuganathan *et al*., 2004). Thus, while lipid peroxidation is involved in the toxicity of some metals, such as Cd, there are other metals, such as Cr, which do not involve lipid peroxidation.

Proteins are also subject to oxidative damage and are major components of biological systems. ROS generated in the cell are capable of directly reacting with amino acid side chains in proteins. The products of metal catalyzed oxidation of proteins are listed in Table 1 (Shacter, 2000) and range from disulfides to carbonyls. The sulfur containing amino acids, cysteine and methionine, are most susceptible to oxidation. They form disulfides and methionine sulfoxide,

Table 1: Products of metal catalyzed oxidation in proteins

Modifications	**Amino acids involved**
Disulfides, glutathiolation	Cys
Methionine sulfoxide	Met
Carbonyls (aldehydes and ketones)	All
Oxo-histidine	His
Dityrosine	Tyr
Cross-links, aggregates and fragments	Several

respectively upon oxidation. Other amino acids are also oxidized. Table 2 lists the products of metal-catalyzed oxidation of a few such amino acids (Shacter, 2000).

Among side chain modifications considered part of protein oxidative modifications, the most common and easily detectable one is carbonylation. Amino acids such as Lys, Arg, Pro and Thr incur formation of carbonyl groups (aldehydes and ketones). As shown in Figure 3, carbonyl groups in proteins can be derivatized with 2, 4 dinitrophenyl hydrazine (DNPH), which can in turn be detected using an antibody directed against the DNP group or by using ELISA. 2, 4-DNPH binds to oxidized proteins not only qualitatively but also quantitatively, making this assay spectrophotometrically quantifiable as well.

Proteins are not only modified by ROS, but can also be covalently modified by products of oxidative stress. Thus, as shown in Figure 2, proteins are also oxidized by by-products of lipid peroxidation (such as malonyldialdehyde and 4-hydroxynonenal), by aberrant proteins newly synthesized from damaged DNA and RNA as well as by oxidized proteins themselves. Oxidation of amino acid side chains and fragmentation of the peptide backbone can lead to major structural and conformational change in proteins. Oxidation of amino acids in proteins increases their hydrophilicity, resulting in exposure of amino acids that would otherwise remain buried within the interior of the protein (Chao et al., 1997; Lasch et al., 2001). Proteins also incur formation of inter-molecular, intra-molecular or mixed disulfides through their Cys residues. Such physical changes either directly or subsequently result in protein unfolding, dimerization, cross-linking and aggregation. Since oxidized proteins undergo structural and conformational changes, they are “earmarked” for proteolysis, and selectively degraded by proteases. The 20S - proteasome

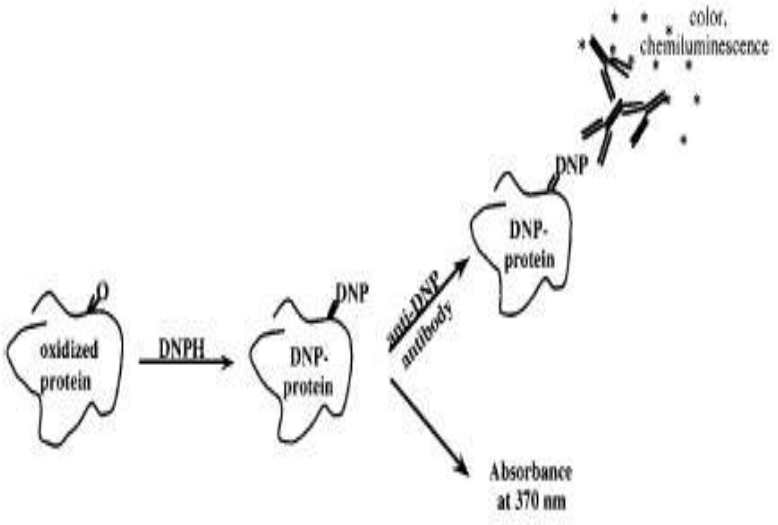

Figure 3. Identifying oxidized (carbonylated) proteins

Oxidized proteins can be identified using DNPH derivatization as an approach for detecting oxidized proteins by immunochemistry or by spectrometry (Shacter, 2000)**.**

Table 2: Metal catalyzed amino acid modifications in proteins

Amino acid oxidized	**Product(s)**
Histidine	Aspartate, asparagines, oxo-histidine
Proline	Hydroxyproline, glutamate, γ-glutamylsemialdehyde
Lysine	Amino-adipicsemialdehyde
Arginine	γ-glutamylsemialdehyde
Threonine	Amino-ketobutyrate
Tyrosine	Tyr-Tyr (dityrosine)
Cysteine	-S-S- (disulfide cross-links)
Methionine	Methionine sulfoxide

dependent clearing of oxidized proteins is meant to prevent the accumulation of damaged, aberrant and misfolded oxidized proteins. Such a clearing away of oxidized proteins results in a decrease in the protein levels, or their abundance, as was seen in vascular endothelial cells, wherein the levels of the oxidized protein, alpha-tubulin, was seen to decrease over time in response to metal-catalyzed oxidation (Bernhard *et al*., 2005). Even so, some cross-linked aggregates are resistant to 20S proteasome clearance and inhibit the functions of the proteasomic machinery, leading to damage and death (Widmer and Grune, 2005). Protein aggregation (metal induced or otherwise) is responsible for the pathogenicity of neuro-degenerative disorders and is an active area of study.

Accompanying and as a consequence of, these major physical changes, and as a consequence of which, biological function of the protein is also compromised (Oliver *et al*., 1987). Indeed, there are reports of many enzymes showing a decrease in enzyme activity following oxidation. One such study in aging houseflies specifically shows that aconitase, a mitochondrial enzyme, decreases in activity following oxidation (Dayan and Paine, 2001). Another study in aging erythrocytes shows that activities of glyceraldehyde-3-P-dehydrogenase, aminotransferase and phosphoglycerate kinase all decreased with oxidation (Oliver *et al*., 1987). The effect on enzyme activity can range from mild to severe depending on the percentage of molecules modified and the duration and/or persistence of damage (Shacter, 2000). Such loss in function following oxidation is seen not only in enzymes but also in structural proteins. Cu ion exposure results in the oxidation of LDLs (low-density lipoproteins) and the subsequent aggregation of the protein (Yang *et al*., 1997).

Metal induced damage to microtubules, particularly α-tubulin has also been shown to occur in vascular endothelial cells (Bernhard *et al*., 2005).

Proteins are the most abundant macromolecules, whether at the level of cell, tissue or biological fluid (Grune and Davies, 1997). Moreover, the reactivity of the most potent among the ROS, hydroxyl radical ($OH^{\cdot}$), is greatest towards proteins, and varies over a very small range between the different macromolecules (Buxton, 1988). Since the reactivity (or rate of the reaction) is the product of the rate constant and the concentration of the target, there is, consequently, a higher potential for greater damage to proteins from ROS (Grune and Davies, 1997). Studies undertaken to predict the sites of damage in leukocytes induced by singlet oxygen and hydroxyl radical (based upon kinetics and abundance data) showed that approximately 70% of the damage occurred at the site of proteins as shown in Figure 4, (Grune and Davies, 1997). Furthermore, studies show that protein oxidation is mechanistically involved in the pathogenicity of a number of diseases including atherosclerosis, neuro-degenerative disorders, ischemia-reperfusion, liver cirrhosis and cancer (Stadtman and Berlett, 1998). Also, protein oxidation and damage have been shown to be the principal routes of toxicity -at least for metals, such as Cr (Sumner *et al*., 2005). And yet, in the field of oxidative stress, protein oxidation has not been well addressed, and remains little understood. The complexities of the target, as well as the paucity (until recently) of stable and sensitive methods to study protein oxidation, are possibly some of the reasons for protein oxidation being the "poor cousin" to lipid and DNA oxidation.

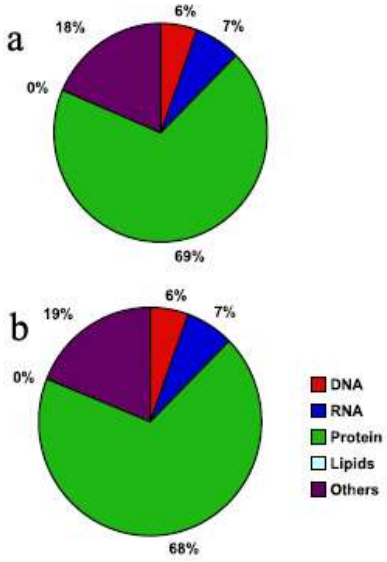

Figure 4: Predicted sites of damage induced by ROS in leukocytes

Kinetic and abundance data were used to predict the sites of damage in leukocytes induced by singlet oxygen (a) and hydroxyl radical (b) (Grune and Davies, 1997).

The implication of protein oxidation in the pathogenicity of a wide variety of diseases, taken together with the fact at least for some metals protein oxidation is the principle route to toxicity and is one of the earliest changes observed in a number of pathologies, makes protein oxidative damage and protein responses pertinent aspects to study and forms the focus of this dissertation.

Experimental questions

Protein oxidation and damage in response to oxidative stress in general and metal induced oxidative stress in particular are not well characterized. It is known that metal exposure leads to an increase in protein carbonyls, indicative of protein oxidation (Avery *et al*., 2000). However, whether or not the damage is targeted towards particular proteins has not been fuly investigated. Furthermore, most metal toxicity studies choose to study oxidative damage sustained by the cell after prolonged metal exposure. While these studies characterize the after-effects of metal toxicity, they fail to identify the means and mechanisms of metal toxicity. It is in fact the early, or immediate, responses that potentially decide the fate of the cell. And yet, there are very few studies that focus on studying these early responses. We address this concern in our study by choosing to study protein oxidation sustained by the cell at different time points within an hour of metal exposure, since previous studies indicated protein carbonyl content to be maximal between 15 and 45 minutes after metal treatment (Avery *et al*., 2000). Moreover, since different metals such as Cu, Cd and Cr are known to elicit oxidative stress and exert toxicity through different mechanisms, it is unknown whether or not their protein oxidation response would be conserved. With regard to protein oxidation, oxidative stress and metal toxicity several questions remain.

1. Would oxidative damage be random or targeted to specific proteins in the cell?
2. Would the protein oxidation response be conserved among the different metals?
3. What are the functional consequences of protein oxidative damage?

The results and answers to these questions and implication of the results constitute Chapters 1-5 of this dissertation.

Significance

Proteins are essential macromolecules and are critical for cellular function. Also, protein oxidation is being considered a principal route of metal toxicity. Studies show that protein oxidation is not only involved in the pathogenicity of a number of diseases including, but not limited to, atherosclerosis and Alzheimer's disease, but also is the earliest manifestation in the pathogenicity (Davies, 1995; Ding *et al*., 2006 and Poppek and Grune, 2006). Therefore, characterizing the effects of metal exposure on the proteins of the cell is vital to furthering our understanding of the molecular mechanisms behind the etiology and progression of these diseases.

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CHAPTER 1

Copper-induced Oxidative Stress in *Saccharomyces cerevisiae* Targets Enzymes of the Glycolytic Pathway

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Increased cellular levels of reactive oxygen species (ROS) are known to arise during exposure of organisms to elevated metal concentrations, but the consequences for cells in the context of metal toxicity are poorly characterized. Using two-dimensional gel electrophoresis, combined with immunodetection of protein carbonyls, we report here that exposure of the yeast *Saccharomyces cerevisiae* to copper causes a marked increase in cellular protein-carbonyl levels, indicative of oxidative protein damage. The response was time-dependent, with total-protein oxidation peaking approximately 15 minutes after the onset of copper treatment. Moreover, this oxidative damage was not evenly distributed among the expressed proteins of the cell. Rather, in a similar manner to peroxide-induced oxidative stress, copper-dependent protein carbonylation appeared to target glycolytic pathway and related enzymes, as well as heat shock proteins. Oxidative-targeting of these and other enzymes was isoform-specific and, in most cases, was also associated with a decline in the proteins' relative abundance. Our results are consistent with a model in which copper-induced oxidative stress disables the flow of carbon through the preferred glycolytic pathway, and promotes the production of glucose-equivalents within the pentose phosphate pathway. Such re-routing of the metabolic flux may serve as a rapid-response mechanism to help cells counter the damaging effects of copper-induced oxidative stress.

Introduction

Copper is one of the essential trace elements, required as a cofactor for a number of enzymes and other cellular activities, including the uptake of iron (Fernandes et al., 2002; Linder and Hazegh-Azam, 1996; O'Halloran and Culotta, 2000; Stearman et al., 1996). In excess, however, copper is toxic and potentially carcinogenic. Neurodegenerative disorders, autism and Alzheimer's disease are some of the conditions that have been linked with copper exposure in humans (Ciriolo et al., 1994; Kawanishi et al., 2002; Rottkamp et al., 2000; Wecker et al., 1985). It is widely considered that copper exerts its effects at the cellular level at least in part through induction of oxidative stress (Avery, 2001; Cadenas et al., 1989; Dormer et al., 2000; Santoro and Thiele, 1997). Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O^{\cdot}_2^-$), and hydroxyl radical ($OH^{\cdot}$) arise normally during aerobic metabolism (with an estimated 2% of oxygen utilized by the yeast cell being converted into the superoxide anion) (Richter and Schweizer, 1997). However, ROS have the potential to cause oxidative damage to proteins, nucleic acids and other macromolecules, which can severely compromise cell health and viability (Halliwell and Gutteridge, 1999). Of necessity, therefore, aerobic organisms (eukaryotic and prokaryotic) have developed a network of defense mechanisms to protect against ROS. These include ROS-scavenging molecules (e.g., superoxide dismutases, catalases), oxidative damage-repair enzymes (e.g., methionine sulfoxide reductase) (Avery, 2001; Cadenas et al., 1989; Dormer et al., 2000; Santoro and Thiele, 1997), and mechanisms such as the S-thiolation of oxidation-susceptible proteins, which prevents oxidation by forming reversible mixed-disulphide bonds with glutathione/thiols (Shenton and Grant, 2003). Environmental stresses such as irradiation and exposure to heavy metals are known to promote ROS formation in cells, potentially overwhelming antioxidant defenses. This may be

particularly true of redox-active metals, like copper, which catalyze the Fenton reaction and can accelerate generation of the highly damaging OH° radical from $O°_2^-$ and H_2O_2 substrates (Avery, 2001; Halliwell and Gutteridge, 1999). Oxidative damage to lipid membranes, for example, has previously been identified as one mode of copper action in the yeast model *Saccharomyces cerevisiae* (Avery et al., 1996; Howlett and Avery, 1997a). Proteins are also major targets of ROS in cells, either through oxidation of their amino acid side chains to hydroxy- or carbonyl-derivatives, or by a shearing of their peptide bonds (Cabiscol *et al*., 2000; Costa *et al*., 2002). Moreover, individual proteins may display differing susceptibilities to oxidative attack, linked to variable compositions of sulphydryl groups, Fe-S clusters, reduced haeme moieties and Cu prosthetic groups (Grune and Davies, 1997). Recently there has been a considerable effort to characterize the major cellular protein targets of ROS. A number of reports have attempted to address this issue in yeast, by determining some of the proteins that were either oxidatively-damaged or specifically modified (S-thiolated) by treatment with H_2O_2 (Cabiscol *et al*., 2000; Costa *et al*., 2002; Shenton and Grant, 2003). The assay for protein carbonyl content is particularly useful since this modification records relatively accurately the fraction of oxidatively-damaged protein with impaired function in total protein samples (Requena *et al*., 2001). In this report we ascertain, for the first time, which proteins are specifically carbonylated in the presence of subcritical, growth-inhibitory levels of copper. We found that there were marked temporal differences in the targeted oxidation of susceptible proteins during copper treatment, and that copper-dependent protein oxidation was isoform-specific. Moreover, the results indicate that a re-routing of metabolic flux likely occurs in cells exposed to non-lethal levels of copper, which may serve as a protective response to copper-dependent oxidative stress.

Materials and methods

Strains, media and copper exposure

Saccharomyces cerevisiae BY4741 (*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was routinely maintained on YEPD agar. Experimental cultures in YEPD broth were inoculated from 24-h starter cultures derived from single colonies and grown overnight to exponential phase (OD_{600} ~2.0) at 30 °C with orbital shaking (120 rev min^{-1}) (Avery, 2001). At the start of experiments, copper nitrate $Cu(NO_3)_2$ was added to flasks to a final concentration of 8 mM and cultures were incubated with shaking as above. Growth was monitored with optical density at 600 nm. For determination of cell viability, aliquots of cell suspension were removed, diluted and plated on YEPD agar. Colony forming ability was determined after 4 days at 30°C. At intervals during copper exposure, cell samples were harvested by centrifugation and flash-frozen.

Preparation of protein extracts

Frozen cell pellets were resuspended in lysis buffer (100 mM Tris, pH7.4, 10% (v/v) glycerol, 1 mM PMSF, 264 mg ml^{-1} aprotinin, 20 mg leupeptin, 10 mg ml^{-1} pepstatin). Cells were disrupted with glass beads (0.5 mm diameter) using a mini-bead-beater (Biospec Products), interspersed with cooling on ice. Cell debris was removed by centrifugation (12,000 x *g*, 10 min, 4 °C) and supernatants retained for protein analyses. Protein concentrations in the supernatants were determined according to Bradford (1976).

Two dimensional gel electrophoresis and Western blot analysis

The general methods for 2D protein analysis and Western blotting are adapted from Ausubel *et al*. (1992-2002) with modifications for immunodetection of protein carbonyl groups

(Conrad et al., 2000; Costa et al., 2002). For 2D-analysis either 40 µg (for protein detection) or 100µg (for Western blotting) of protein was mixed with re-hydration solution (8M urea, 0.4% DTT, 4% CHAPS and 1% IPG buffer, pH 3-10) and loaded onto 18 cm immobilized pH gradient (IPG) strips (non-linear pH gradient 3-10; Amersham Pharmacia). Iso-electric focusing was performed according to the protocol of Gorg *et al.* (2000). Strips containing samples intended for Western blotting were derivatized with 2,4-dinitrophenylhydrazine (DNPH) by equilibration in 10 ml of 10 mM DNPH in 0.2 N HCl for 20 min. All protein-laden strips were then equilibrated in SDS buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol for 15 min, and containing 4% iodoacetamide for an additional 15 min. Proteins within the strips were resolved further by electrophoresis through 12.5% homogenous polyacrylamide gels. For visualization of total proteins (40 µg-protein samples), the 2D protein arrays were stained overnight with SYPROruby™ fluorescent protein-gel stain, as recommended by manufacturer (Molecular Probes). Fluorescence from the proteins was imaged and quantified using the 2D Master Imaging System and Decyder analysis software 4.0 (Amersham Pharmacia). For analysis of carbonylated proteins by Western blotting, the derivatized proteins were electroblotted onto PVDF membranes. The membranes were incubated in PBS-Tween containing 5% (w/v) skimmed milk powder, and were probed with rabbit anti-DNP as primary antibody (Molecular Probes Inc; 1:16,000 dilution) and peroxidase-linked goat anti-rabbit IgG as secondary antibody (Sigma; 1:16,000 dilution). Carbonylated proteins were immunodetected with a chemiluminescent peroxidase substrate, West femtoM (Pierce) (Conrad et al., 2000), and visualized using a Fuji LAS1000 Image Analyzer with pre-cooled camera. Chemiluminescence was quantified using Fuji Image gauge software.

Identification of proteins

For preliminary determination of proteins after 2D-resolution, images from the SYPROruby™ stained gels were compared to the 2D yeast proteome database (*http://www.ibgc.u-bordeaux2.fr/YPM/)* and the Swiss-2D-PAGE database (*http://www.expasy.org/images/swiss-2dpage/publi/yeast-high.gif*) (Gorg *et al*., 2000). The identity of specific proteins within the protein array that were shown to be susceptible to carbonylation was confirmed by sequence analysis. Proteins of interest were excised from 2D gels that had been stained with 0.05 % (w/v) Coomassie blue in 0.5% (v/v) acetic acid, 20% (v/v) methanol and subsequently destained with 30% (v/v) methanol. The excised proteins were digested overnight with trypsin (11 ng/µl), and the masses of the resulting peptides analyzed by MALDI-mass spectrometry (Voyager DE Pro; Applied Biosystems). Identification of each sequence was based on sequences available in the SWISS-PROT protein database (Costa *et al*., 2002).

Results

Copper exposure causes protein oxidation in cells

A $Cu(NO_3)_2$ concentration of 8 mM was selected for copper-treatment experiments. At this concentration the mean cell doubling time was increased by ~10% compared to non-Cu-treated controls, but there was no discernible loss of cell viability (colony forming ability; data not shown), indicating that protein extracts were representative of all the copper-treated cells in cultures. A previous study indicated that protein oxidation in response to stress typically peaks within 15 to 45 min (Avery *et al*., 2000). Consequently, our analyses focused primarily on the period 0 to 60 min after copper treatment. Immuno-chemiluminescent labeling for detection of

oxidized/carbonylated proteins revealed that copper caused rapid, but transient, oxidation of total-soluble proteins with isoelectric points in the range of 3 to 10 (the range that was tested) (Figure 5). Total carbonyl levels in these cellular proteins were unaltered after 5 min of copper treatment, but were increased by approximately 8-fold after 15 min. There was a subsequent decline in carbonylated proteins until around 60 min, when the levels of oxidized proteins were again similar to those of untreated cells. There were no significant changes in total-protein oxidation in parallel, untreated control flasks (data not shown).

Preferential copper-dependent oxidation of enzymes of the glycolytic pathway and of heat shock proteins

To gain insight into the oxidation of individual proteins at various times during copper treatment, cellular proteins from the relevant extracts were separated in a two dimensional array, according to their pI and mass. Figure 6A depicts a typical 2D profile of proteins from exponential phase yeast cells incubated without copper. The arrangement and relative abundances of proteins displayed corresponds well with standard yeast 2D protein-profiles produced in other laboratories (see *http://www.expasy.org/images/swis-2dpage/publi/yeast-high.gif* *http://www. ibgc.u-bordeaux2.fr/YPM/*), in which proteins have been systematically identified by mass spectrometry (Fauchon *et al*., 2002). Neither the expressed-protein profiles nor the corresponding profiles of carbonylated proteins varied significantly during the 60-min time course with non-Cu-treated control cells (data not shown). While the expressed protein-profile for cells that had been exposed to 8 mM copper for 30 min (Figure 6C) was similar overall to that of non-exposed cells (Figure 6A), enhanced copper-dependent protein oxidation was evident from the increased intensity and numbers of carbonylated proteins in immunoblots derived from extracts of copper treated cells (Figure 6B and Figure 6D).

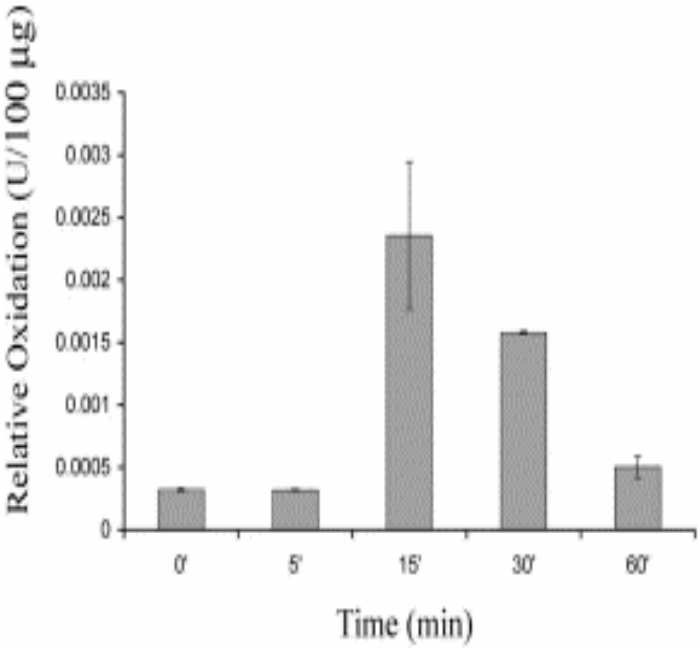

Figure 5: Total protein oxidation during copper exposure.

Protein extracts were prepared from cells at intervals during treatment with 8 mM $Cu(NO_3)_2$. 2D-Western blots were probed with anti-DNP antibodies and visualized with a chemiluminescent substrate to identify carbonyl groups. Oxidation was quantified using Fuji Image gauge software. Total oxidation was calculated according to the sum of the chemiluminescence of all proteins in the entire blot (after background-correction), and by normalizing this value against the total protein loaded onto each gel. Values reflect results from two or more 2D gel analyses, and are depicted as such in the Figure, along with their standard deviation from the mean.

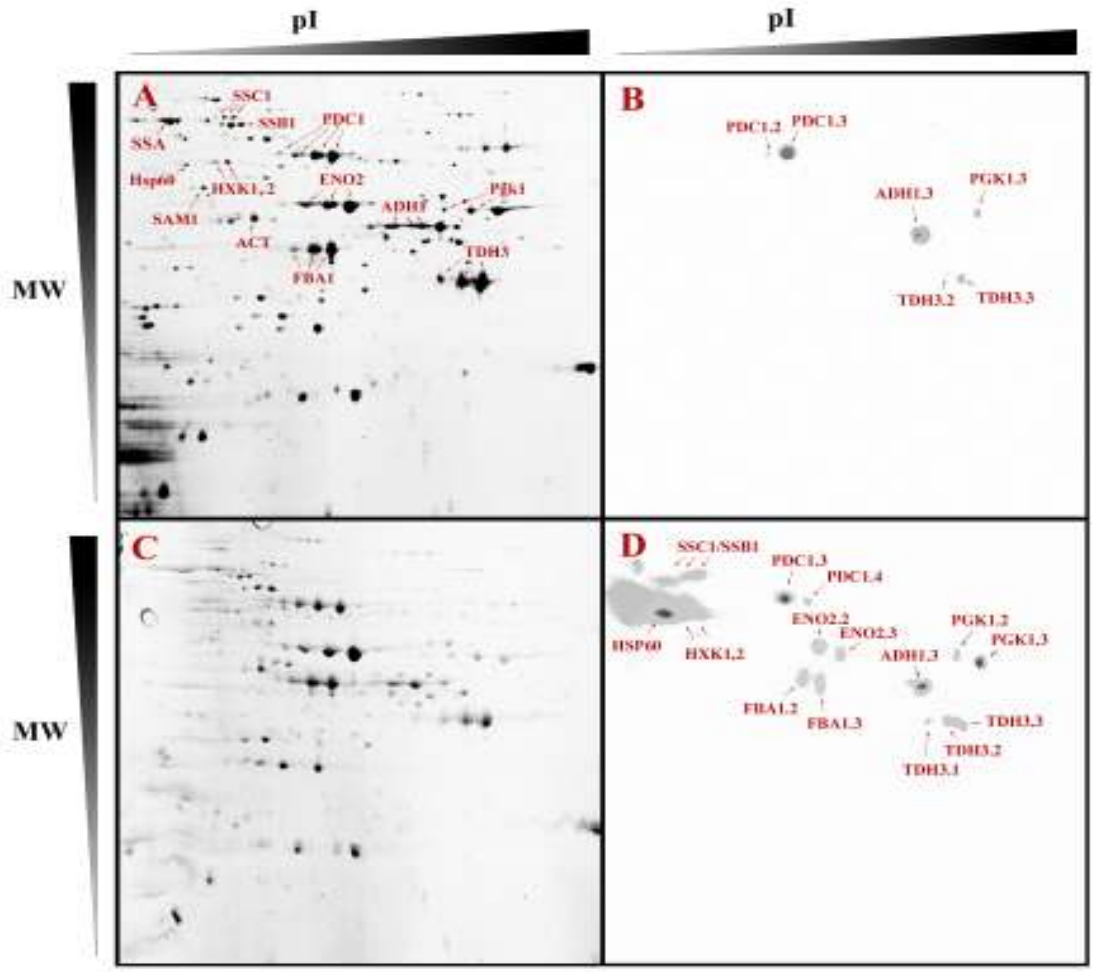

Figure 6: 2D-profiles of protein abundance and oxidation during copper exposure.

Protein extracts were prepared from cells just prior to Cu exposure (Panels A and B) or after 30 min treatment with 8 mM $Cu(NO_3)_2$ (Panels C and D). Panels A and C depict 2-Dimensional arrays of cellular proteins stained with SYPRO-ruby. Panels B and D depict Western blots of arrayed proteins probed with anti-DNP antibodies to identify oxidized/carbonylated proteins. Gels shown are typical of the results obtained from various 2-Dimensional protein separations prepared from cell extracts at different times after cells were exposed to copper. The relative position of proteins that were shown to be susceptible to oxidation are referenced in panels A, B and D by their standard abbreviations.

Proteins-of-interest that were detected in immunoblots over the time course of copper exposure were characterized further. In all cases, the distributions of proteins in gels and blots were in excellent agreement with each other, and with the available 2D gel databases (see Materials and Methods). Nevertheless, the identity of each protein-of-interest was validated by MALDI-mass spectrometry (Table 3). Strikingly, of the 10 detectable protein-types that were oxidized at some stage during copper treatment, eight were found to be involved either directly in glycolysis or in subsequent catabolic reactions (Table 3; Figure 7). The marked susceptibility of heat shock proteins (HSPs) to oxidation was also apparent (Figure 6D; Table 3). Indeed, the extent of HSP oxidation after 15 minutes or more precluded any consistent resolution of the oxidation levels for any of the individual proteins (exemplified in Figure 6D). Consequently these proteins were treated as a family of proteins, and their collective abundance and oxidation profiles are reported as such (Table 3). The proximity of these heat shock proteins on the 2D Western blots to Hexokinase 1 (Hxk1), Hexokinase 2 (Hxk2) and S-adenosylmethionine synthetase (Sam1) also prevented any determination of the oxidized levels of these enzymes after 15-30 minutes, effectively nullifying any temporal evaluation of their susceptibility to oxidation.

In order to characterize each of the proteins that were most susceptible to copper-dependent oxidation at each time point, data for individual proteins from each gel and Western blot were normalized against the total soluble-protein concentration, quantified on SYPROruby™-stained gels at each time point. In so doing, it was apparent that different proteins exhibited marked differences in copper-dependent carbonylation, both in degree and in time-dependence (Figure 7). This diversity of oxidative effects on individual proteins is reflected in the fact that only one protein (Adh1) exhibited a pattern of oxidation that closely

Table 3. Proteins that are carbonylated within sixty minutes of 8 mM copper exposure.

No.	Std. Protein Name	Name	MW	pI
1	Act1	Actin	41.7	5.19
2	Adh1	Alcohol dehydrogenase 1	36.8	6.06
3	Eno2	Enolase 2	46.8	5.56
4	Fba1	Fructosebiphosphate aldolase 1	39.5	5.39
5	Tdh3	Glyceraldehyde-3-phosphate dehydrogenase 3	35.6	6.5
6	Hxk1 & 2 *	Hexokinase 1 & 2 *	53.6	5.07
7	HSPs*	Heat shock proteins* (HSP60, SSA1, SSA2, SSB1, SSC1)		
8	Pdc1	Pyruvate decarboxylase 1	61.4	5.62
9	Pgk1	Phosphoglycerate kinase 1	61.2	5.89
10	Sam1	S-adenosylmethionine synthetase 1	41.7	4.79

* Denotes a protein or series of proteins which exhibited varying degrees of carbonylation, but the degree of oxidation was difficult to denote/differentiate throughout the time course of the experiment.

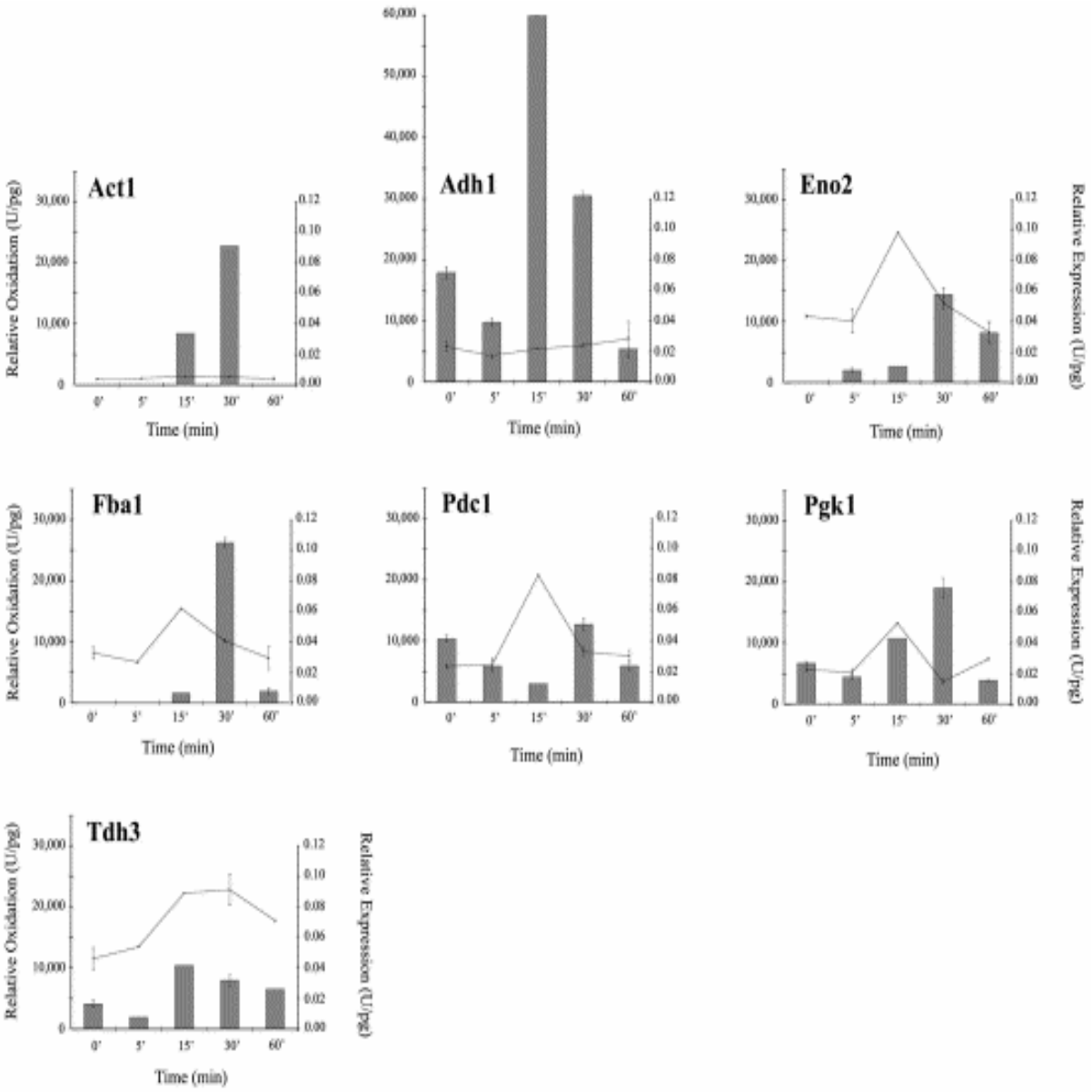

Figure 7: Oxidation levels and abundances of individual proteins during copper exposure.

The oxidation levels (histograms) and protein abundances (lines) of each targeted protein were quantified in extracts prepared from cells at intervals during exposure to 8 mM $Cu(NO_3)_2$. Owing to the inherent limits of 2D Western analysis, data for the heat shock proteins, hexokinase and S-adenosylmethionine synthetase are not presented here. The oxidation levels shown for individual proteins are normalized against their relative abundance at the corresponding time points, and subsequently expressed as a function of total protein. Values reflect results from two or more independent gel separations and are depicted along with their standard deviation.

resembled the averaged trend seen for the total protein (Figure 5 and Figure 7). Several other proteins, however, also showed significant increases in oxidation after the cells had been exposed to copper, namely: Fba1, Eno2, Pdc1, Tdh3, Pgk1 and Act1. Among these, only Tdh3 exhibited a peak of carbonylation after 15 min, whereas maximal oxidation of the other proteins was evident only after 30 min (Figure 7). Curiously, the abundance of individual proteins (relative to total loaded protein) often exhibited an increase after 15 min, before returning to pre-copper levels after 60 min. The effect was most striking for Eno2 and Pdc1, each of which exhibited a >2.5-fold change in relative abundance during this time (Figure 7). In contrast, levels of Act1 and Adh1 (Figure 7), Hxk1, Hxk2, Sam1 and the HSPs (data not shown) were relatively constant throughout.

Differing susceptibilities of individual isoforms of protein-targets to copper-dependent oxidation

With the exception of Hxk1, all the glycolysis-related enzymes that were targeted for carbonylation during copper stress were present in the cell extracts as three or more distinct isoforms, whose profile could be discerned on 2D gels (Figure 6). The relative abundance of the individual isoforms of each protein and their apparent susceptibilities to copper-dependent oxidative damage differed markedly. Thus, for example, the peak level of carbonylation was clearly greater for Adh1.1 and Pgk1.1 than for the other detected isoforms of these two proteins (Figure 8). Moreover, with the possible exception of Adh1, the most abundant isoform of each enzyme consistently appeared to be the one that was targeted least by copper-dependent oxidation. By way of example, mean levels of Eno2.3 were >2-fold higher than those of either

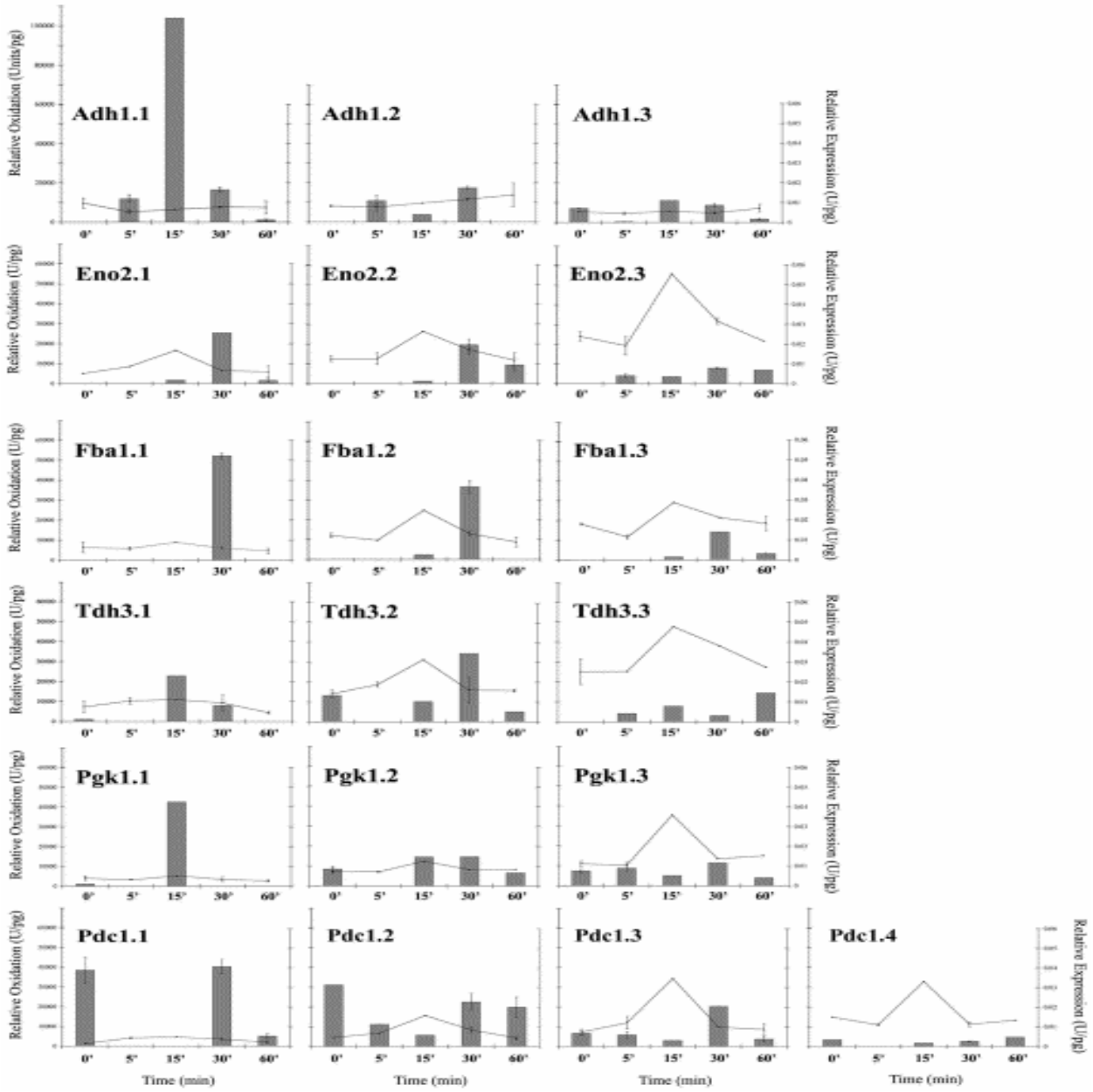

Figure 8: Oxidation levels and abundances of individual isoforms of targeted proteins during copper exposure.

The oxidation levels (histograms) and abundances (lines) of each targeted protein were quantified in extracts prepared from cells at different intervals during exposure to 8 mM $Cu(NO_3)_2$. The oxidation levels shown for individual isoforms are normalized against their relative abundance at the corresponding time points, and subsequently expressed as a function of total protein. Values shown reflect results from two or more independent gel separations (whenever possible) and are depicted along with their standard deviation.

Eno2.1 or Eno2.2 during the time course examined, whereas peak carbonylation of Eno2.3 was >2-fold lower than in the other isoforms.

Discussion

In this study we demonstrate that exposing *Saccharomyces cerevisiae* to sub-lethal levels of copper inflicts oxidative damage on a number of its cytoplasmic proteins. The level of oxidation peaked within 15 to 30 minutes after copper addition, establishing a temporal component to copper-induced protein oxidation. The majority of proteins that were oxidized over the course of the investigation also demonstrated an increase in their relative abundance after 15 minutes of exposure. The subsequent decline in oxidation after 15 minutes was coincident with the maximal oxidation levels of most copper-targeted proteins. The transient peak in levels of oxidized proteins was similar to previous observations (Avery *et al*., 2000), and consistent with selective degradation of oxidatively-damaged proteins (Costa *et al*., 2002). Such a correlation, however, was not always observed in this investigation. Adh1 (the most heavily oxidized enzyme of all those identified) showed no evidence of altered levels of expression or degradation throughout the investigation, indicating that protein degradation need not be a necessary consequence of excessive or directed oxidation.

This study also demonstrated that, in a similar manner to peroxide-induced oxidative stress, copper treatment resulted in the specific oxidation/carbonylation of a discrete subset of predominantly catabolic enzymes. While the targeted gene products also included abundant proteins, such as actin and several heat shock proteins, the majority of proteins which demonstrated a clear and specific susceptibility to oxidation during copper exposure were

enzymes involved either directly in glycolysis, or in the fermentation of the glycolytic product, pyruvate. A similar targeting specificity has recently been observed during peroxide-induced oxidative stress in *Saccharomyces cerevisiae* (Cabiscol *et al*., 2000; Shenton and Grant, 2003), wherein, low levels of hydrogen peroxide elicited the preferential damage and inhibition of a more limited set of glycolytic enzymes. These similarities add to the previously documented overlaps in other aspects of the copper- and oxidative-stress responses of yeast (Avery, 2001; Cadenas et al., 1989; Dormer et al., 2000; Santoro and Thiele, 1997) and lend further support to the hypothesis that oxidative mechanisms underpin copper toxicity (Avery, 2001).

Among the catabolic enzymes shown here to be susceptible to copper-dependent oxidation, all except Hxk, Pgk1 and Pdc1 have also been shown to be targets for protein S-thiolation, an activity that serves to protect proteins from irreversible oxidative damage (Shenton and Grant, 2003). This association between susceptibility to oxidation and preferential S-thiolation persists even among isoenzymes of a single protein species. Shenton and Grant (2003) demonstrated that of the three different isoenzymes of glyceraldehyde 3-P-dehydrogenase expressed in *S. cerevisiae* (Tdh1, Tdh2 and Tdh3), only Tdh3 was specifically modified by S-thiolation. Similarly, in the present study, copper-induced oxidation was detectable only in Tdh3 and not in either Tdh1 or Tdh2, despite the presence of the latter two isoenzymes in the protein array. While it may appear logical that oxidation-susceptible proteins should be the ones that merit specific protection mechanisms, it is noteworthy that the interplay of such opposing actions (specifically, oxidative damage versus scaleable protection against oxidative damage) provides a rather classic regulatory mechanism for a finely controlled cellular response to oxidative stress. This regulatory interplay is all the more plausible, given the collective and individual dynamics

of the oxidation profile of the different proteins within the first sixty minutes after copper exposure (Figure 5 and Figure 8), and the time (approximately one hour) after which oxidative damage and S-thiolation of these proteins was demonstrated in the hydrogen peroxide stress induced cells (Costa *et al*., 2002; Shenton and Grant, 2003).

The oxidation of Adh1 affords a mechanism by which fermention of glucose, the preferred metabolism of yeast grown on glucose (Gasch and Werner-Washburne, 2002), can be rapidly influenced. This, along with the apparent, directed oxidation of the other glycolytic pathway enzymes, is consistent with a cellular metabolic response to other forms of oxidative stress that has been promoted by a number of groups (Costa *et al*., 2002; Ravichandran *et al*., 1994; Schuppe-Koistinen *et al*., 1994; Shenton and Grant, 2003). This response involves a transient metabolic re-shuffling of glucose equivalents through the pentose phosphate pathway resulting from the targeted inactivation of specific glycolytic enzymes. It has been proposed that this metabolic shift could provide the necessary reducing power ($NADPH_2$) for antioxidant enzymes such as those of the glutaredoxin system (Cabiscol et al., 2000; Holmgren, 1989; Lee et al., 1999). In addition, the directed oxidation of glycolytic enzymes may temporarily alleviate the repressive effects of glucose metabolism on cellular antioxidant defense mechanisms, such as the transcriptional induction of catalase, superoxide dismutase and/or glutathione peroxidases (Krems *et al.,* 1995; Moradas-Ferreira *et al*., 1996). Thus, even in cells using glucose as a sole carbon source, a transient cessation of glucose metabolism might facilitate cellular defense against oxidative stress. While the oxidative data is incomplete, a similar argument can be made for the preferential oxidation of the only non-glycolytically related enzyme that was shown to be oxidized in this analysis, S-adenosylmethionine synthetase (Sam1; Table 3). Given the

importance of glutathione as an antioxidant, and its presumed role in metal resistance in yeast (Izawa et al., 1998; Vido et al., 2001), a transient inactivation of Sam1 by oxidation could serve to divert the sulphur amino acid biosynthetic pathway away from the formation of S-adenosyl homocysteine and toward the production of glutathione. Whatever the mechanism(s), directed oxidation of the glycolytic and fermentative enzymes as well as Sam1 seems likely to be beneficial to cells in countering copper-induced oxidative stress.

The precision that is apparent in the targeting of specific metabolic enzymes during copper exposure is even more clearly demonstrated by the differing susceptibilities of individual isoforms of these enzymes to oxidation. Similar isoform-specific responses have been reported previously. For example, apolipoprotein E in oxidatively-stressed human cells exhibits isoform-specific free-radical scavenging activity (Jolivalt *et al*., 2000). In regard to copper-induced oxidation, it is of particular interest that the most-abundant isoform of each enzyme was the least affected by copper-dependent oxidation (Figure 8). This is even more remarkable when one considers that (with the notable exception of Adh1) all the targeted catabolic enzymes experience a significant increase in their relative abundance within 15 minutes after exposure to copper (Figure 7). While the precise association of protein oxidation and its effect upon the function of each enzyme still need to be determined, the multimeric nature of the targeted enzymes may provide a clue as to how oxidation of one isoform could have an impact upon the stability of another, and thus the function of the intact enzyme. Indeed, were the *transient* reduction of glycolytic activity to be the goal (resulting in a transitory re-routing of carbon units through the pentose phosphate pathway), then perhaps oxidation of the least abundant isoform would provide an ideal means to disrupt enzyme function, while still retaining a significant proportion of

unmodified subunits to allow for the subsequent rapid restoration of optimal glycolytic metabolism. Further work is required to substantiate this additional, possible layer of targeted response to copper stress.

Whatever the underlying mechanisms or consequences of protein oxidation, the results shown in this study clearly demonstrate that exposure of *S. cerevisiae* to sub-lethal levels of copper induces an oxidative stress that is similar in scope and specificity to that induced by hydrogen peroxide and other reactive oxygen species. Furthermore, the results also indicate that this copper-induced oxidative stress occurs within the first hour of exposure, and is a dynamic process that specifically targets key enzymes involved in the fermentative catabolism of glucose.

Acknowledgements

This research was supported by grants from the National Institutes of Health (GM579450), the Georgia Research Alliance and Georgia State University (RPE awards).

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CHAPTER 2

Oxidative Protein Damage causes Chromium Toxicity in Yeast

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Oxidative damage in microbial cells occurs during exposure to the toxic metal chromium, but it is not certain whether such oxidation accounts for the toxicity of Cr. Here, a *Saccharomyces cerevisiae sod1Δ* mutant (defective for the Cu, Zn-superoxide dismutase) was found to be hyper-sensitive to Cr(VI) toxicity under aerobic conditions, but this phenotype was suppressed under anaerobic conditions. Studies with cells expressing a Sod1p variant ($Sod1^{H46C}$) showed that the superoxide dismutase activity rather than the metal-binding function of Sod1p was required for Cr-resistance. To help identify the macromolecular target(s) of Cr-dependent oxidative damage, cells deficient for reduction of phospholipid hydroperoxides (*gpx3Δ,* and *gpx1Δ/gpx2Δ/gpx3Δ)* and for the repair of DNA oxidation (*ogg1Δ*, and *rad30Δ/ogg1Δ*) were tested, but were found not to be Cr sensitive. In contrast, *S. cerevisiae msraΔ* (*mxr1Δ*) and *msrbΔ* (*ycl033cΔ*) mutants defective for peptide methionine sulfoxide reductase (MSR) activity exhibited a Cr-sensitivity phenotype, and cells overexpressing these enzymes were Cr resistant. Overexpression of MSRs also suppressed the Cr sensitivity of *sod1Δ* cells. The inference that protein oxidation is a primary mechanism of Cr toxicity was corroborated by an observed ~20-fold increase in the cellular levels of protein carbonyls within 30 min of Cr exposure. Carbonylation was not

distributed evenly among the expressed proteins of the cells; certain glycolytic enzymes and heat shock proteins were specifically targeted by Cr-dependent oxidative damage. This study establishes an oxidative mode of Cr toxicity in *S. cerevisiae,* which primarily involves oxidative damage to cellular proteins.

Introduction

All metals at elevated concentrations are potentially toxic, whether or not they are biologically essential at more moderate levels (Gadd, 1992). Metal toxicity can be readily observed as inhibition of growth or metabolic activity in metal-treated microorganisms, and metal exposure of higher organisms is associated with a range of harmful effects; for example, chromium exposure has been linked to genotoxicity, carcinogenicity, and allergenicity (Dayan and Paine, 2001). These biological consequences of metal exposure at the tissue or whole organism level are well characterized. However, there is a poor understanding of the mechanism(s) of toxicity of metals such as chromium at the cellular or molecular level, and this needs to be addressed.

Chromium is a highly toxic non-essential metal, which is used in the production of steel and other alloys, in metal finishes, and leather tanning. Waste from such processes (in addition to the high natural abundance of Cr) has made Cr a serious environmental pollutant. It is widely hypothesised that toxicity due to Cr (as well as many other metals) may arise due to enhanced generation of reactive oxygen species (ROS) and oxidative damage in Cr-exposed organisms. Cr, like other redox-active metals, may catalyze Fenton-type reactions to promote free radical formation (Halliwell and Gutteridge, 1999). However, most of the existing evidence that links oxidative processes to Cr toxicity is correlation-based, typified by enhanced oxidation in Cr-exposed organisms. While valuable, such evidence has not helped to resolve whether such oxidation effects are actually important for Cr toxicity. One reason for the absence of more robust evidence is the experimental limitations imposed on studies of this nature by animal models. The budding yeast *Saccharomyces cerevisiae* provides an attractive alternative system

for elucidating the mechanism(s) of metal toxicity (Avery, 2001). Moreover, Cr toxicity towards yeasts and other microorganisms is of interest in its own right, both from environmental and biotechnological perspectives (Cervantes et al., 2001; White et al., 1998).

Cr exists primarily in the Cr(III) and Cr(VI) oxidation states, the latter hexavalent species being considered the more toxic in the environment due to its higher solubility and mobility. Cr(VI) accumulated by organisms is reduced to Cr(III) with the concomitant production of intermediate Cr(V) and Cr(IV) products and oxygen- and carbon-based radicals (Ackerley *et al*., 2004; Cervantes *et al*., 2001; Pourahmad and O'Brien, 2001). These species are known to be associated with a spectrum of DNA lesions occurring during Cr(VI) exposure (Aiyar et al., 1991; Luo et al., 1996; Pourahmad and O'Brien, 2001; Reynolds et al., 2004), many of which are oxidative in nature. However, a quadruple *apn1/rad1/ntg1/ntg2* mutant of *S. cerevisiae,* which is impaired in the repair of abasic sites in DNA and is hypersensitive to oxidizing agents such as menadione and hydrogen peroxide (Swanson *et al*., 1999), did not display hypersensitivity to Cr(VI) (Pourahmad and O'Brien, 2001). Protection against Cr by ROS-scavenging molecules and other antioxidants has been reported in several organisms (Pesti *et al*., 2002; Pourahmad and O'Brien, 2001). In *S. cerevisiae,* Cr has been shown to affect mitochondrial function (Fernandes et al., 2002; Henderson, 1989), and the antioxidant protein alkyl hydroperoxide reductase (Ahp1p) protects against Cr toxicity (Nguyen-Nhu and Knoops, 2002). However, while generally this type of evidence is consistent with a role for ROS in Cr toxicity, it is not necessarily demonstrative. For example, many antioxidant molecules or proteins have non-specific activities (e.g. metal-binding as well as ROS-scavenging functions), making it difficult to assign associated phenotypes with antioxidant properties specifically (Avery, 2001).

In this paper we use the power of yeast genetics to test whether oxidative mechanisms are a cause of Cr toxicity in *S. cerevisiae.* In particular, we exploit the specificity of engineered Cu,Zn-superoxide dismutase (Sod1) variant proteins and of cellular oxidative damage-repair systems to show that Cr toxicity is oxidative in nature, with cellular proteins being primary targets.

Materials and Methods

Strains and plasmids

Saccharomyces cerevisiae BY4741 (*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and derivative deletion mutants *sod1*Δ*, gpx1*Δ*, ogg1*Δ*, msra*Δ (*mxr1*Δ) and *msrb*Δ (*ycl033c*Δ) were obtained from Euroscarf (Frankfurt, Germany). An isogenic *gpx1Δ/gpx2Δ/gpx3Δ* triple mutant was constructed previously (Avery & Avery, 2001). A *msraΔ/msrbΔ* mutant in the same background (BY4741) and the plasmid YEp351-MsrA (Kryukov *et al*., 2002) were kindly provided by V. N. Gladyshev (University of Nebraska). *S. cerevisiae* EMY74.7 and the derivative double-deletion strain *ogg1Δ/rad30Δ* (Haracska *et al*., 2000) were gifts from Dr. L. Prakash, University of Texas. The plasmids YEp351-Sod1 (YEp600) (Nishida et al., 1994) and YEp351-Sod1^{H46C} (Gorg et al., 2000; Li et al., 1997)were kindly provided by E. B. Gralla (University of California). All plasmids were transformed into *S. cerevisiae* according to Gietz & Woods (2002). The plasmid YEp352-MsrB was constructed in this study after PCR-amplification from yeast genomic DNA of a fragment comprising the full *MSRB* open reading frame together with 600bp of upstream non-coding sequence (primer sequences are available on request). The amplified fragment was ligated between the *Sac*I and *Kpn*I restriction sites of the

vector YEp352, using standard recombinant DNA protocols (Ausubel *et al*., 2004). Sequence fidelity of the cloned PCR product was confirmed by automated DNA sequencing.

Culture conditions and assessment of Cr toxicity.

Yeast strains were routinely maintained on YEPD agar or, for plasmid maintenance, on YNB agar supplemented with the appropriate amino acids or nucleic acid bases (Ausubel *et al*., 2004). For experiments, organisms were cultured at 30ºC, 120 rev. min^{-1} to mid-/late-exponential phase (A_{600} ~2.0) either in liquid YEPD, or in YNB medium for experiments involving plasmid-bearing strains. For enumeration of viable cells on CrO_3-supplemented agar, the experimental cultures were adjusted to OD_{600}~0.0004 in sterile YEPD broth and 200 µl aliquots were plated onto YEPD agar supplemented with CrO_3 at the desired concentration. Colony forming ability was determined after 8 d at 30^{o}C.

For spotting experiments, experimental cultures were each adjusted to A_{600} of ~2.5, 0.25, 0.025, 0.0025 and 0.00025. Samples (4 µl) from each dilution were spotted on YEPD agar, supplemented with CrO_3 as specified. Growth was examined after incubation for 4 d at 30ºC. Where specified, plates were incubated anaerobically in an N_2 atmosphere. For experiments involving linolenic acid (18:3), media were prepared with tergitol (Nonidet P-4-; Sigma) to solubilize the fatty acid and 1 µl aliquots were spotted from dilution series starting at OD_{600} ~5.0. The final tergitol concentration was 1% (wt/vol); tergitol has no adverse effect on yeast growth (Howlett and Avery, 1997a).

Analysis of protein oxidation

At intervals during exposure to 0.5 mM CrO_3 in liquid medium, cell samples were harvested by centrifugation and flash-frozen. Protein extracts were prepared from the cells as outlined previously (Shanmuganathan *et al*., 2004). The methods for 2D protein separation and Western blotting were exactly as described (Shanmuganathan *et al*., 2004). Briefly, proteins were loaded onto immobilized pH gradient strips [pH 3-10; Amersham Pharmacia (or pH 4.5-5.5 for identification of heat shock proteins)] and, after isoelectric focusing, protein carbonyls were derivatized with 2,4-dinitrophenylhydrazine (DNPH). After 2D separation and electroblotting, the derivatized proteins were probed with rabbit anti-DNP as primary antibody (Molecular Probes Inc; 1:16,000 dilution) and peroxidase-linked goat-anti rabbit IgG as secondary antibody (Sigma; 1:16,000 dilution). Carbonylated proteins were immunodetected with a chemiluminescent peroxidase substrate, West femtoM (Pierce) using a Fuji LAS3000 Image Analyzer with pre-cooled camera. Quantification of carbonylated protein and normalization against protein abundance and protein loading were as described previously (Shanmuganathan *et al*., 2004).

For preliminary identification of proteins after 2D-resolution, images from SYPROruby™-(Molecular Probes) stained gels were compared to the 2D yeast proteome databases *www.ibgc.u-bordeaux2.fr/YPM* and *www.expasy.org/images/swis-2dpage/publi/yeast-high.gif*. The identities of specified proteins within the protein arrays were subsequently confirmed by MALDI-mass spectrometry (Voyager DE Pro; Applied Biosystems), as described previously (Shanmuganathan *et al*., 2004).

Results

Chromium toxicity is suppressed by the superoxide dismutation activity of Sod1p

To test whether oxidative mechanisms are involved in Cr toxicity towards *S. cerevisiae,* we investigated first the potential role of the Cu,Zn-superoxide dismutase (Sod1p) in Cr resistance. Due to its high toxicity, Cr(VI) (as CrO_3) was the Cr species tested throughout this study. An *S. cerevisiae sod1Δ* deletion strain was found to be markedly more sensitive to Cr toxicity than wild type cells (Figure 9). Thus, the onset of Cr toxicity (loss of colony forming ability) commenced at approximately 0.23 mM and 0.65 mM CrO_3 for the *sod1Δ* and wild type cells, respectively, with approximately 0.5 mM and 1.1 mM Cr being required to give 90% loss of viability of these strains, respectively (Figure 9).

Sod1p-dependent copper resistance in *S. cerevisiae* was found previously to involve Cu buffering rather than ROS-scavenging by the enzyme (Slekar et al., 1996). There is evidence that Sod1p-binding of zinc (Gorg *et al*., 2000) and of silver (Ciriolo et al., 1994) also underpins Sod1p-dependent resistance to these metals. To determine whether it was the antioxidant activity of Sod1p that conferred Cr resistance, first the dependency of the *sod1Δ* phenotype on aerobic conditions was tested. Anaerobic incubation suppressed the Cr-sensitivity of the *sod1Δ* mutant (Figure 10A). Thus, wild type and *sod1Δ* cells exhibited similar (elevated) resistances to Cr in the absence of oxygen. To substantiate the oxidative mode of Cr toxicity suggested by these results, Cr resistance was further examined in *sod1*Δ cells transformed with plasmids expressing either a wild-type *SOD1* ORF, or a mutant *sod1* allele encoding $Sod1^{H46C}$. $Sod1^{H46C}$ lacks superoxide dismutase catalytic activity but is minimally affected for metal binding capacity (Li et al., 1997). The wild type *SOD1* gene complemented the *sod1*Δ phenotype, restoring

growth in the presence of Cr to wild type levels (Figure 10B). In contrast, Sod1^{H46C} had no influence on the Cr sensitivity of the *sod1Δ* mutant even when expressed on a multi-copy plasmid (YEp351-Sod1^{H46C}), which has been characterised previously (Gorg *et al*., 2000). Together, the results show that it is specifically the antioxidant function of Sod1p that protects *S. cerevisiae* from Cr toxicity.

In order to elucidate the macromolecular targets of oxidative Cr toxicity, a strategy was devised to examine cells defective in the repair of oxidative damage specifically to each of the key macromolecular groups. The yeast protein Gpx3 (PHGpx3/Orp1) encodes a phospholipid hydroperoxidase, reducing phospholipid hydroperoxides (Avery, 2001; Shanmuganathan et al., 2004). Expression of Gpx3 confers resistance in yeast to agents which have a lipid peroxidation-dependent mode of action (Shanmuganathan *et al*., 2004). Assays of lipid peroxidation itself do not resolve whether lipid peroxidation is a/the mode of action. Here, the Cr resistances of a *gpx3*Δ deletion mutant and wild-type *S. cerevisiae* were compared by spotting the strains on Cr-supplemented agar. However, inhibition of the growth of these strains was similar at all Cr concentrations tested (data not shown). Furthermore, a triple *gpx1* Δ/*gpx2* Δ/*gpx3*Δ mutant defective for all three of the yeast phospholipid hydroperoxidase-like enzymes also failed to reveal a Cr-sensitivity phenotype. In support of these results, growth of *S. cerevisiae* in the presence of the oxidation-sensitive polyunsaturated fatty acid (PUFA) linolenic acid, which is incorporated to greater than 60% of total membrane fatty acids under the conditions used (Avery et al., 1996; Howlett and Avery, 1997), did not sensitize cells to Cr (not shown).

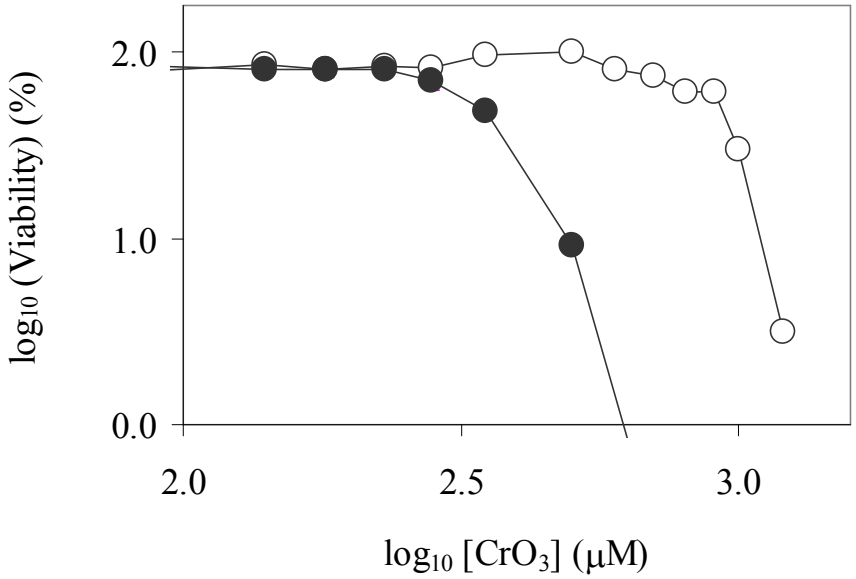

Figure 9. The Cu,Zn- superoxide dismutase protects yeast from Cr toxicity.

Exponential phase cells of *S. cerevisiae* BY4741 (○) and a derivative *sod1Δ* mutant (●) were plated on YEPD agar supplemented with CrO_3 at various concentrations. Viability was determined as colony forming ability after 8 d incubation, and expressed as a percentage of colony forming units (CFUs) determined in control incubations lacking CrO_3. Points correspond to means from at least four separate viability determinations. Error bars representing standard deviations of the mean were smaller than the dimensions of the symbols.

Thus, in contrast to data from equivalent experiments performed with metals such as copper and cadmium (Avery et al., 1996; Howlett and Avery, 1997a; Shanmuganathan et al., 2004), these results collectively indicated that any lipid peroxidation which arises during Cr exposure of yeast is not required for Cr toxicity.

The 8-oxoG DNA glycosylase Ogg1p specifically repairs oxidative DNA damage, 8-oxoG being a critical mutagenic lesion (Kasprzak, 2002). Here, wild type and *ogg1*Δ cells were compared for Cr sensitivity, but no difference was apparent (data not shown). We obtained the same result also with an *ogg1*Δ*/rad30*Δ double mutant, which is susceptible to a synergistic increase in oxidative DNA lesions (Haracska *et al*., 2000) and in pro-oxidant sensitivity (Willets, 2004) versus the *ogg1*Δ and *rad30*Δ single mutants. The results indicated that DNA oxidation arising during Cr exposure is unlikely to be a primary cause of Cr toxicity in yeast (see also Discussion).

To determine whether protein oxidation was involved in the mechanism of Cr toxicity, cells defective for the peptide methionine sulfoxide reductases MsrA and MsrB were tested. These MSR enzymes have complementary activities, reducing different stereoisomers of oxidized methionine (Met) residues in proteins (L-methionine-S-sulfoxide and L-methionine-R-sulfoxide, respectively) (Kryukov *et al*., 2002). MSRs provide the only characterized protein oxidation repair activity of cells. Single *msra*Δ and *msrb*Δ mutant strains exhibited marked sensitivity to Cr compared to wild-type cells, and this phenotype was accentuated in a double *msra* Δ*/msrb*Δ mutant (Figure 11A, B). Thus, the colony forming ability of *msra*Δ */msrb*Δ cells was diminished by approximately three orders of magnitude at 0.8 mM CrO_3, a concentration at

which colony formation by wild type cells was barely affected (Figure 11B). To determine whether the MSRs may be broadly required for metal resistance in yeast, the growth of *msraΔ/msrbΔ* single and double mutants was also tested in the presence of $Cu(NO_3)_2$ (10–16 mM) and $Cd(NO_3)_2$ (50–200 μM), but these strains were no more sensitive to Cu or Cd than the wild type (data not shown).

To substantiate the role for protein (Met) oxidation in Cr toxicity that was suggested by the above data, the *MSRA* and *MSRB* genes were overexpressed: whereas gene deletion can lower the threshold of resistance to an agent by sensitizing a new principal cellular target to that agent, only genes that help to protect the normal target(s) of toxicity (or that directly encode that target) should raise the lower resistance threshold (Shanmuganathan *et al*., 2004). There appeared to be some cell density-dependence of Cr resistance. Thus, growth of individual colonies was accentuated at the highest dilutions tested. Nonetheless, overexpression of the *MSRA* and *MSRB* genes on multicopy plasmids gave markedly enhanced growth of *S. cerevisiae* at this normally inhibitory Cr concentration (Figure 11C). In addition, overexpression of *MSRA* and *MSRB* suppressed the Cr-sensitivity phenotype of *sod1*Δ cells (Figure 11D), suggesting that the enhanced Cr toxicity seen in this mutant (Figure 9 and 10) also is dependent on protein (Met) oxidation.

Cr exposure causes protein oxidation, targeting glycolytic enzymes and heat shock proteins

If, as the above results showed, protein oxidation is important for Cr toxicity, then this should be reflected also by increased levels of protein oxidation during Cr exposure.

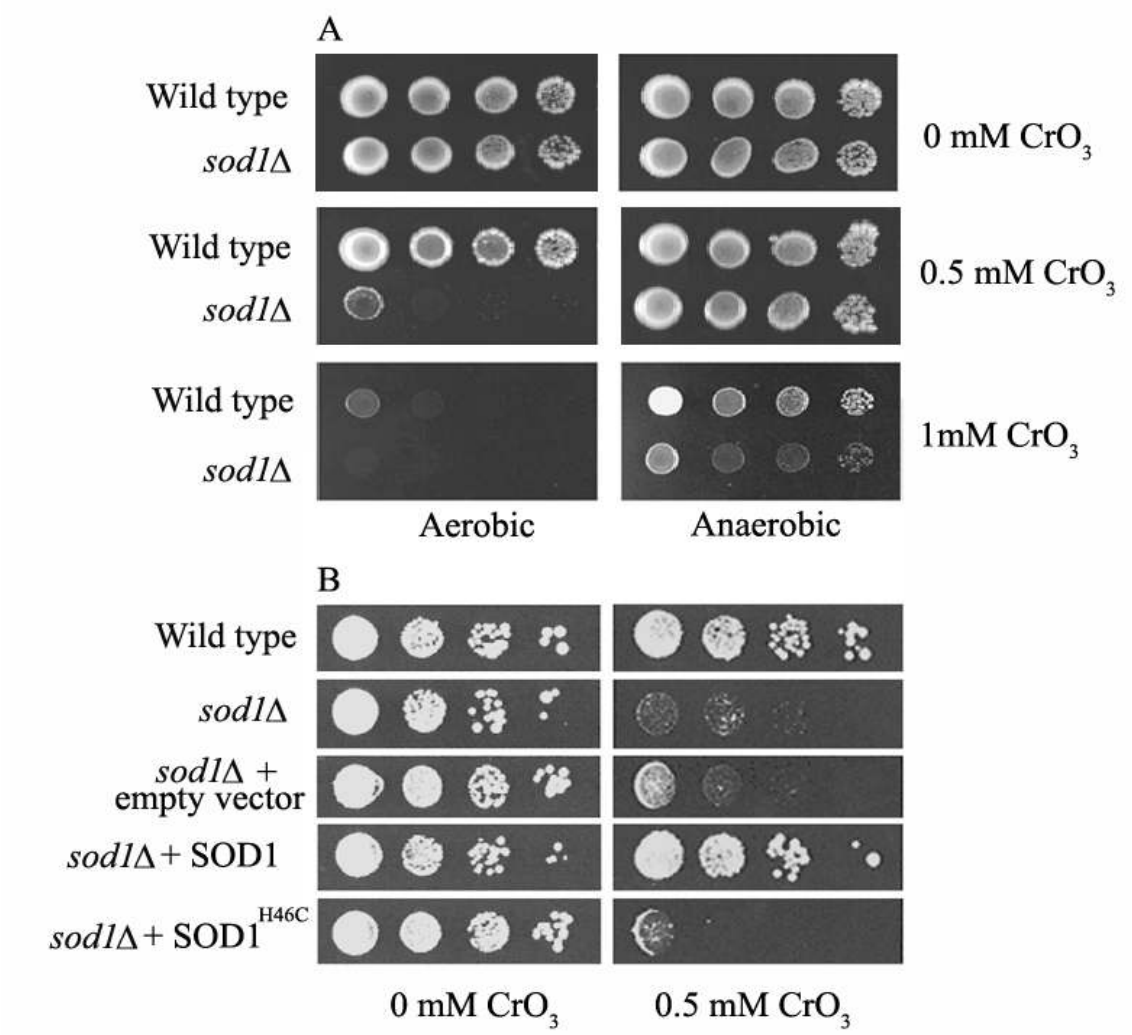

Figure 10. Chromium toxicity is suppressed by anaerobicity and by the superoxide dismutation activity of Sod1p.

Dilutions of decreasing cell concentration (from OD_{600} ~2.5 to ~0.0025) of exponential phase *S. cerevisiae* were inoculated in 4 µl spots from left to right onto YEPD agar supplemented or not with CrO_3. (A) Plates inoculated with *S. cerevisiae* strains BY4741 and a derivative *sod1Δ* mutant were incubated either aerobically, or anaerobically under N_2. (B) Plates supplemented or not with 0.5 mM CrO_3 were inoculated with *S. cerevisiae* BY4741 or a derivative *sod1Δ* mutant and incubated aerobically: where indicated, *sod1Δ* cells were transformed with the vector YEp351 ('+empty vector'), with the same vector harbouring either the wild type *SOD1* gene ('+*SOD1*') or the mutant $SOD1^{H46C}$ gene ('+$SOD1^{H46C}$'). Growth was assessed after 4 d growth. Typical results from one of three independent experiments are shown.

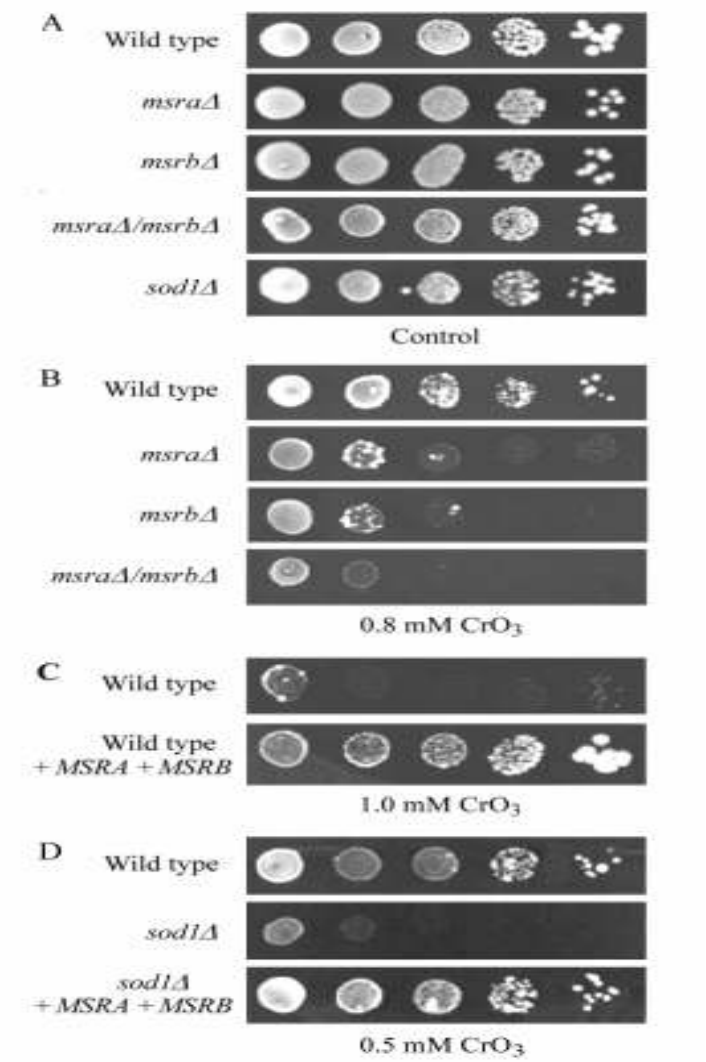

Figure 11. Peptide methionine sulfoxide reductase (MSR) activity is critical for chromium resistance.

Dilutions of decreasing cell concentration (from OD_{600} ~2.5 to ~0.00025) of exponential phase *S. cerevisiae* were inoculated in 4 µl spots from left to right onto YEPD agar, either supplemented or not with 0.5, 0.8 or 1.0 mM CrO_3 as indicated. Plates were inoculated with *S. cerevisiae* BY4741 (wild type) and either derivative *msraΔ, msrbΔ*, *msraΔ/msrbΔ* or *sod1Δ* mutants (A, B). Additional plates were inoculated with wild type or *sod1Δ* cells overexpressing the *MSRA* and *MSRB* genes on multicopy plasmids (‘*+MSRA +MSRB’)* (C, D). Growth was assessed after 4 d incubation. Typical results from one of two independent experiments are shown.

Protein oxidation during Cr exposure has not been examined previously to our knowledge, so protein oxidation was monitored here before and during incubation of cells in the presence of CrO_3, by immunodetection of protein carbonyls (Cabiscol *et al*., 2000; Costa *et al*., 2002; Shanmuganathan *et al*., 2004). The assay of protein carbonyl content is particularly useful since this modification reports relatively accurately on the fraction of oxidatively-damaged protein with impaired function in total protein samples (Requena *et al*., 2001). Note that oxidized Met does not contribute to the protein carbonyl signal, so determination of protein carbonyls provided independent corroboration of protein oxidation (cf. data in Figure 11). A non-lethal concentration of 0.5 mM CrO_3 was selected for these experiments, so that protein extracts were representative of all the Cr-treated cells in cultures: a higher dose may give rise to membrane permeabilisation and protein leakage from some cells but not others, yielding non-representative protein extracts. Chromium caused rapid but transient oxidation of total soluble proteins (Figure 12A; proteins with pI in the range of 3 to 10 were examined). Increased protein carbonyl levels were evident within 5 min of Cr exposure. Most Cr-dependent protein oxidation occurred between 15 and 30 min, after which time total carbonyl levels were approximately 20-fold higher than those of control cells that were not exposed to Cr. There was a subsequent decline in the level of protein carbonylation, but this index of protein oxidation was still approximately 10-fold higher after 60 min than that of cells before Cr-exposure. There were no significant changes in total-protein oxidation in parallel, untreated control flasks (not shown).

To test whether particular protein targets may become highly carbonylated during Cr treatment, proteins from cells that had been exposed to Cr for different times were extracted and separated according to their pI and mass using 2D gels. The arrangement and relative

abundances of proteins in SYPROruby™-stained 2D gels for each time point (0 and 60 min shown in Figure 12B) corresponded well with standard profiles for *S. cerevisiae* available in 2D protein gel databases (see Materials and Methods). The identity of each protein-of-interest was validated by MALDI-mass spectrometry. Cr-dependent protein oxidation was evident from the increased intensity and numbers of carbonylated proteins in immunoblots derived from extracts of chromium-treated cells (Figure 12B). Five of the proteins that became most highly oxidized during Cr treatment were cytosolic enzymes involved either directly in glycolysis or in subsequent catabolic reactions: Fba1 (fructose biphosphate aldolase), Eno2 (enolase), Hxk1 and Hxk2 (hexokinases 1 and 2) and Pdc1 (pyruvate decarboxylase) (Figure 12B; Figure 13). Different isoforms of these enzymes showed differing susceptibilities to Cr-induced carbonylation. In addition, mitochondrial, cytosolic and nuclear heat shock proteins (HSPs) including Hsp60, Ssa1,2, Ssb1 and Ssc1 exhibited very marked carbonylation during Cr exposure, which was sufficiently intense that these proteins' proximities within the 2D arrays precluded clear and consistent resolution of their individual levels of oxidation.

Proteins similar to those found here for Cr were reported previously to be carbonylated during exposure of *S. cerevisiae* to other pro-oxidants, including another toxic metal Cu (Shanmuganathan *et al*., 2004). Since the cause of Cu toxicity does not appear to involve protein oxidation (see above, and Discussion), it was of interest to ascertain whether particular proteins were selectively targeted by Cr versus Cu. To facilitate comparison, carbonylation data obtained previously at sub-lethal Cu concentrations (Shanmuganathan *et al*., 2004) were normalized against the equivalent data for Cr (see legend to Figure 13). Thus, differences were apparent in the relative carbonylation of individual proteins during Cr and Cu exposure (Fig. 5).

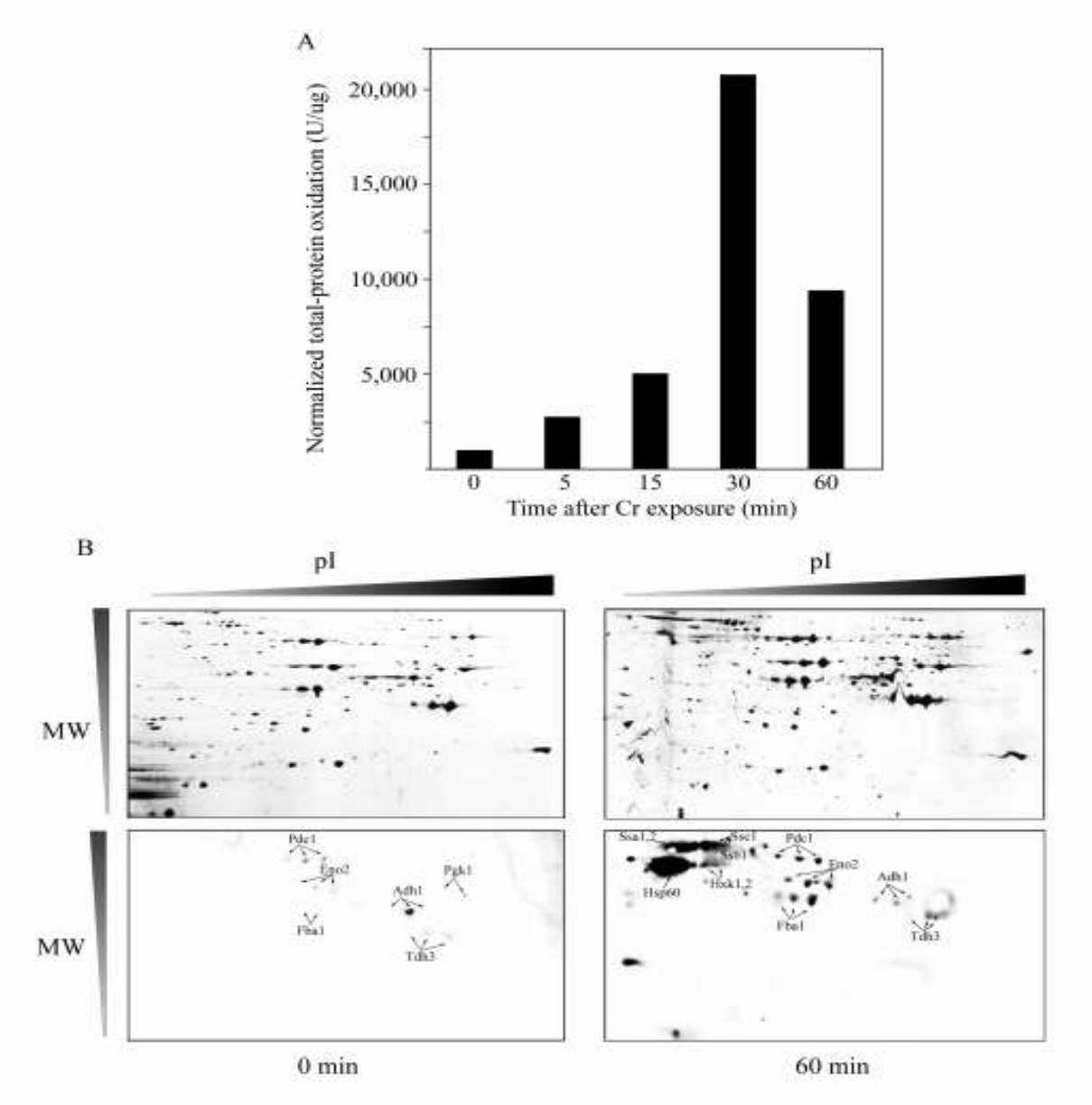

Figure 12. Chromium exposure is associated with increased levels of oxidized proteins.

Protein extracts were prepared from *S. cerevisiae* BY4741 at intervals during treatment with 0.5 mM CrO_3. 2D-Western blots were probed with anti-DNP antibody and visualized with a chemiluminescent substrate to identify carbonylated (oxidized) proteins. (A) Total levels of protein oxidation over time were calculated after normalization, as described previously (44). (B) Representative two-dimensional arrays of cellular proteins stained with SYPRO-ruby (top panels) and corresponding western blots (lower panels) are shown for protein extracts sampled at 0 min and 60 min during CrO_3 treatment. The positions of oxidation-susceptible proteins are referenced.

Tdh3 and Adh1 tended to be more susceptible to Cu-dependent oxidation, relative to the other proteins examined. In contrast, Eno2, Fba1 and Pdc1 were more strongly affected by Cr treatment (data for individual HSPs could not be resolved, for the reasons stated above). Collectively, these results indicate that Cr causes rapid yet selective oxidation of cellular protein targets in *S. cerevisiae.* Such selectivity is noteworthy given the importance of protein oxidation for Cr toxicity established in this paper.

Discussion

This paper demonstrates an oxidative mechanism of Cr(VI) toxicity, which involves protein oxidation. Several previous studies have indicated a correlation between levels of oxidation in cells and Cr exposure, although a causal link with Cr toxicity has proved more elusive. A principal reason for this, in addition to the correlation-based nature of certain evidence, is the non-specificity for ROS of many 'antioxidant' gene products that have been manipulated to address this problem [for review, see Avery (2001)]. Here, a Sod1p variant that was defective specifically for superoxide dismutase catalytic activity (Gorg *et al*., 2000; Li *et al*., 1997) was used to substantiate that this was the activity responsible for the strong *SOD1*-dependency of Cr resistance in *S. cerevisiae,* as demonstrated also by the aerobic requirement of the Cr hyper-sensitivity phenotype of *sod1*Δ cells. It can be inferred that Cr exposure generates superoxide at toxic levels.

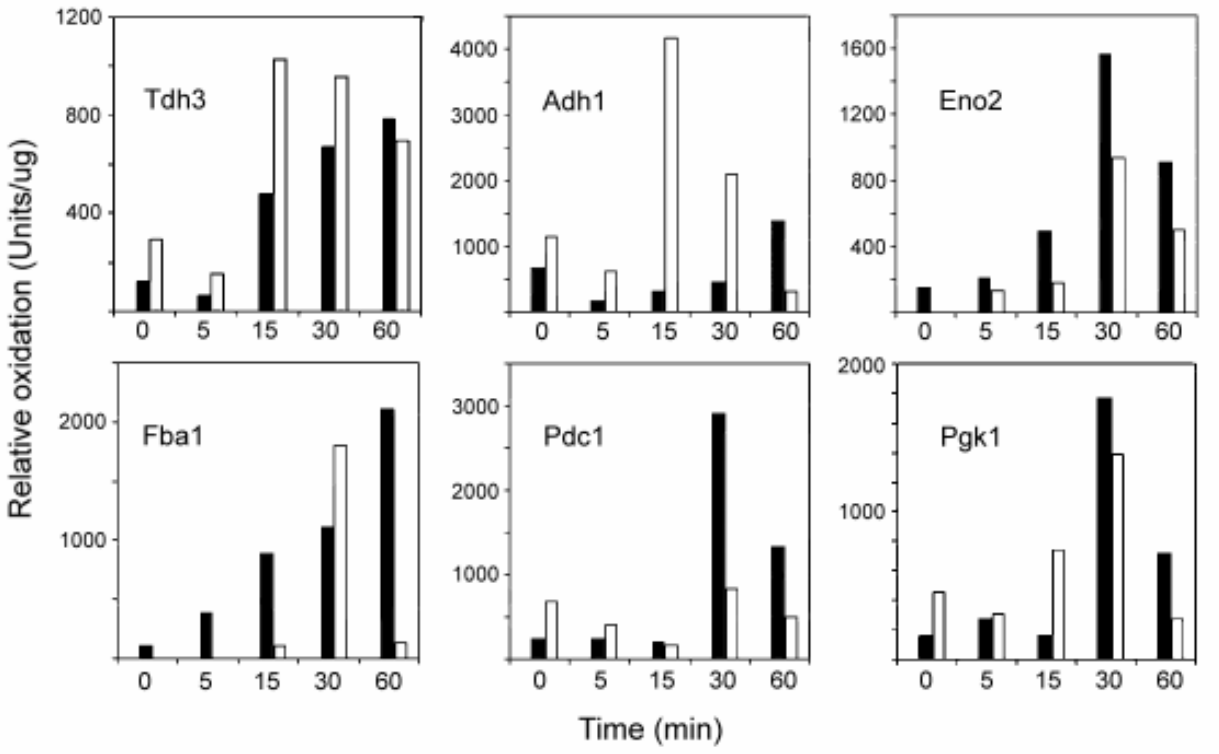

Figure 13. Oxidation levels of individual proteins during chromium exposure.

The oxidation levels of targeted proteins were quantified in extracts prepared from cells at intervals during exposure to 0.5 mM CrO_3. Owing to inherent resolution limitations of the 2D western analysis, data for the HSPs and Hxk1,2 are not presented here. The oxidation levels for individual proteins during Cr exposure (■) are normalized against their relative abundance at the corresponding time points, and subsequently expressed as a function of total protein. Data for individual isoforms were combined for each protein. For comparison of relative susceptibilities of individual proteins to Cr- and Cu-mediated oxidation, carbonyl data for proteins from cells treated with a sub-lethal concentration of 8 mM $Cu(NO_3)_2$ were normalized against those with Cr; i.e. each datum for Cu was multiplied by a factor obtained by dividing the mean carbonylation value across all samples and proteins with Cu by the equivalent mean with Cr (□ = copper data).

Cell cycle- and age-dependent fluctuations in Sod1p have been found to drive variation in the copper resistances of individual cells of *S. cerevisiae* (Sumner et al., 2003). Here, the similar gradients (albeit non-superimposed) of Cr dose-response curves for wild type and *sod1Δ* cells (Fig. 1) together with a relatively weak cell cycle dependency of Cr resistance (data not shown) indicated that, while Sod1p is critical for culture-averaged Cr resistance, it does not appear to drive cell-to-cell heterogeneity in Cr resistance. This different effect to that seen with Cu, combined with the fact that Cu-binding by Sod1p confers Cu resistance (Slekar et al., 1996) whereas the enzyme's superoxide dismutase activity gives Cr resistance (this study), is consistent with these differing functions of Sod1p being subject to distinct regulatory controls.

Phospholipid hydroperoxide glutathione peroxidases (PHGPxs) are capable of reducing phospholipid hydroperoxides in biological membranes and so they repair oxidative damage, specifically, to membrane lipids. In yeast cells, expression of the PHGPx-like enzymes Gpx1-3 confers resistance to agents which have a lipid peroxidation-dependent mode of action (Avery, 2001; Shanmuganathan et al., 2004). This was exploited here to show that Cr toxicity towards *S. cerevisiae* does not require lipid peroxidation. This outcome was different to that obtained recently with another metal, cadmium (Shanmuganathan *et al*., 2004). In that study, Cd resistance in *S. cerevisiae* was shown to depend specifically on the phospholipid hydroperoxidase activity of Gpx3p, dissected away from other Gpx3p-dependent activities such as transduction of redox-stress signals (Delaunay *et al*., 2002). Evidently, none of the Gpx3p-dependent activities (including phospholipid hydroperoxidase activity) are important for Cr resistance. Consistent with this, enrichment of *S. cerevisiae* with an oxidation-sensitive PUFA

did not sensitize the cells to Cr, unlike the outcome found previously for Cu and Cd (Avery et al., 1996; Howlett and Avery, 1996). In addition, the adverse effects of Cr on yeast mitochondrial function were found elsewhere to occur in the apparent absence of lipid peroxidation (Fernandes et al., 2002).

Results obtained with cells defective for Ogg1p, a protein important for repairing oxidative DNA specifically, in conjunction with other evidence (Pourahmad and O'Brien, 2001); see below], indicated that DNA oxidation is not a primary cause of Cr toxicity. Oxidation of guanine residues is a major form of DNA damage arising from metal-induced oxidative stress (Kasprzak, 2002). Other forms of DNA damage are known to arise during cellular exposure to elevated Cr(VI) concentrations (e.g. Cr-DNA interstrand crosslinks (Pourahmad and O'Brien, 2001; Reynolds *et al*., 2004), and it cannot be ruled out that selective formation of oxidative DNA lesions other than 8-oxoG could contribute to Cr toxicity. However, Cr treatment does enhance 8-oxoG formation, possibly as a result of diminished Ogg1p activity (Hodges and Chipman, 2002), but just not at a level that causes Cr toxicity according to our results. Similarly, although Cr(VI) causes alternative oxidative lesions to DNA that are repairable by Apn1p in *S. cerevisiae* (Cheng et al., 1998), even in a quadruple *apn1/rad1/ntg1/ntg2* mutant such lesions were not sufficient to elicit a Cr(VI)-hypersensitivity phenotype (Pourahmad and O'Brien, 2001).

With regard to the above conclusions, it is acknowledged that there are overlaps in the induction pathways and activities of certain antioxidant proteins and such proteins may compensate functionally for each other. However, such overlaps are not maintained across the full spectrum of antioxidant gene functions (Dormer et al., 2000) and the differential effects

reported here underscore this point: the specificities of the Gpx, Ogg1 and Msr proteins for repair of oxidative damage to distinct macromolecular groups borne out by previous studies of differential activities and phenotypes associated with the corresponding deletion strains (Haracska *et al*., 2000; Kryukov *et al*., 2002; Shanmuganathan *et al*., 2004; Willets, 2004) enabled us to discriminate between these groups as the candidate toxicity targets of Cr. It is emphasised that the present data refer to functions that protect against the continuous presence of Cr, a situation that may be more likely to be experienced naturally, rather than recovery after a brief Cr stress.

Methionine sulfoxide reductase activity, the only protein oxidation repair activity known in biology, proved to be critical for Cr resistance. This result was particularly compelling since overexpression of the MSR-encoding yeast genes raised the lower threshold of Cr tolerance. In contrast to gene overexpression, gene deletion can alter (lower) the threshold of cellular metal resistance by sensitizing a new target to the metal, i.e. a primary target different to that in wild type cells. Overexpression should be effective in altering (raising) the lower resistance-threshold only with a gene product that helps to protect the normal target(s) of toxicity, or that is the target itself (Shanmuganathan *et al*., 2004).

The observation that manipulation of both yeast MSR-encoding genes gave a stronger phenotype than either gene alone was consistent with certain MSR-dependent phenotypes reported in other studies (Koc *et al*., 2004; Kryukov *et al*., 2002). While MSR enzymes have narrow specificity (for oxidized Met), Met residues are especially susceptible to metal-catalyzed oxidation in proteins (Izawa et al., 1998). This, together with the potentially-critical role of Met residues for function of individual proteins, and the protection of other residues that is

considered to result from oxidation of surface exposed Met (Levine et al., 2000), would explain why MSR expression has the marked impact on Cr resistance evident here. Note that protein oxidation was of course not restricted to Met residues, as demonstrated by the increased protein carbonyl levels observed during Cr exposure; carbonyl groups do not result from Met oxidation but are the main oxidation products of other oxidation-susceptible residues such as Arg, Lys and Pro.

It is known that Cr can bind to proteins and may be associated with enhanced protein degradation (Feng et al., 2003; Shrivastava and Nair, 2000). However, Cr-DNA interactions are also widely reported. Thus, it is emphasized that the current data do not necessarily mean that proteins are more strongly targeted than lipids or DNA by ROS formed during Cr exposure. Rather, our data show that the damage caused by ROS to proteins has greater consequences for whole-cell inhibition than effects on the other macromolecules. That protein oxidation is particularly important for Cr toxicity was also consistent with the fact that Cr exposure gave an approximate 20-fold increase in total carbonyl levels, whereas copper maximally gave only an ~8-fold increase (Shanmuganathan *et al*., 2004), with the metals supplied at just sub-lethal concentrations in both cases. Moreover, MSR activity was found not to affect Cu (or Cd) resistance in the present study. Furthermore, Cu-induced protein oxidation returned to basal levels within 60 min, whereas protein carbonyl levels were still elevated 10-fold after the same period of Cr treatment. The decline in total carbonyl levels between 30 min and 60 min exposure to Cr likely reflects selective degradation of oxidatively damaged proteins (Grune and Davies, 1997).

Whereas the total carbonylation induced by Cr was relatively high, Cr targeted a similar range of yeast proteins as Cu (Shanmuganathan *et al*., 2004) and other pro-oxidants (Cabiscol *et al*., 2000; Costa *et al*., 2002). Enzymes involved in glycolysis or in the fermentation of pyruvate, as well as heat shock proteins were particularly susceptible to oxidative modification. These results are consistent with the hypothesis that the glycolytic pathway may become inactivated during (Cr-induced) oxidative stress, promoting the production of glucose equivalents within the pentose phosphate pathway (Costa *et al*., 2002; Ravichandran *et al*., 1994; Shenton and Grant, 2003). Such re-routing of the metabolic flux is considered to serve as a rapid adaptive response to oxidative stress, since it may provide additional reducing power in the form of $NADPH_2$ necessary for the function of certain antioxidant enzymes (Cabiscol et al., 2000; Lee et al., 1999). Furthermore, re-routing may alleviate glucose repression of antioxidant gene transcription (Costa et al., 2002).

As in studies with other oxidants, detection of the complete spectrum of yeast proteins that are oxidatively targeted by Cr is limited here by the sensitivity of the 2D-carbonyl assay. Nevertheless, the similar pattern of carbonylated proteins to that seen with H_2O_2 further supports an oxidative mode of Cr toxicity. Despite the similar sub-sets of proteins targeted, individual proteins exhibited differing relative susceptibilities specifically to Cr- or Cu-mediated oxidation. The proteins examined (Figure 13) were all primarily cytosolic, suggesting that differential targeting by Cr and Cu is unlikely be a result of differing protein or metal localization. Rather, the proteins may have differing binding affinities for Cr and Cu. Moreover, alongside higher induction of protein oxidation by Cr versus Cu (see above), these results suggested that the differing contribution of protein oxidation to the toxicities of Cr and Cu could also be explained

by some selective targeting of the oxidative protein damage that is associated with each metal. Since this study uniquely establishes protein (methionine) oxidation as a primary cause of Cr toxicity, the 2D-analyses presented also suggest some preliminary candidates - albeit ones for which Met-oxidation specifically has not been measured - for future efforts now to identify the specific protein target(s) of cellular Cr toxicity.

Acknowledgements

This research was supported by a grant from the National Institutes of Health (R01 GM57945). We thank Drs. V. N. Gladyshev (University of Nebraska), E.B. Gralla (University of California) and L. Prakash (University of Texas) for kindly supplying certain of the strains and plasmids used in this research.

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CHAPTER 3

A Carbon-Metabolism Response to Cadmium Stress Enables Glutathione-mediated Cd Resistance

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Several mechanisms are reportedly used by cells to counter the effects of the toxic metal, cadmium, including a sulfur metabolism response that stimulates glutathione production. *(*M. Fauchon *et al*., (2002) Mol. Cell 9: 713-723*)*. Here, we show that cadmium also provokes a carbon-specific metabolic response, which is required for Cd resistance. Using immuno-detection of oxidized proteins, we demonstrated that cadmium (which is redox inactive) induces a transient, targeted oxidation of glycolytically associated enzymes in yeast, similar to that reported previously in cells exposed to redox-active metals. An observed decrease in glycolytic enzyme activity, at sub-lethal Cd concentrations, contrasted with elevated expression and activity of the key pentose phosphate pathway (PPP) enzyme, glucose-6-phosphate dehydrogenase (Zwf1p). The results predicted a response to Cd involving enhanced flow of carbon through the PPP, which was substantiated by a significant increase in cellular reducing power (in the form of NADPH) during Cd exposure. The increased reducing power was further reflected in a concomitant increase in the cellular levels of reduced glutathione (GSH), a major defense molecule against Cd. These cadmium-induced increases in NADPH and GSH were both blocked in PPP mutants, which exhibited a marked sensitivity to Cd. The results reveal that a re-direction

of carbon metabolism occurs in response to cellular Cd exposure, providing a mechanism additional to the sulfur response for enabling GSH dependent Cd resistance.

Introduction

The widespread industrial use of cadmium (Cd) has contributed significantly to its anthropogenic introduction and persistence in the environment. With little to no known biological function (Lane *et al*., 2005), cadmium can be rapidly absorbed from the blood in mammals, and thereafter accumulates in metallothionein-rich organs, thereby increasing its accrual and distribution through the food chain. As an established genotoxin and carcinogen, cadmium's relatively long biological half-life (greater than 30 years in some instances) further heightens its toxic threat. Even though exposure to this transition metal has been directly associated with hyper mutability in yeast (Jin *et al*., 2003), the variety of effects that cadmium elicits within different organisms at different concentrations has made it difficult to identify specific molecular mechanisms of its toxicity (Filipic *et al*., 2006). Unlike many other carcinogenic metals, cadmium is redox inactive, in that it is unable to catalyze directly the Fenton and Harber-Weiss reactions, which produce potent reactive oxygen species (ROS). Nonetheless, cadmium is still considered an effective prooxidant in cells, presumably through its ability to produce ROS indirectly, either by displacing redox-*active* metals such as copper and iron from the active sites of enzymes (Stohs and Bagchi *et al*., 1995) or by selective depletion of the cells' anti-oxidant defences (Fortuniak *et al*., 1996 and Stohs *et al*., 2001). It is this role of cadmium, acting as an oxidative stressor in the model system *Saccharomyces cerevisiae,* that provides a basis for the response reported in this work. The yeast *S. cerevisiae* has been used extensively to study cellular responses to metals and other pro-oxidants, including cadmium.

Transcription factors such as Yap1p and Met4p mediate a series of transcriptional responses to Cd, which involve upregulation of the *GSH1* gene among others (Lee *et al*., 1999 and Dormer *et al*., 2000). Gsh1p catalyzes the rate limiting step in the biosynthesis of glutathione, a thiol metabolite that is essential for Cd detoxification (Li *et al*., 1997 and Vido *et al*., 2001). Another outcome of Cd-induced transcriptional reprogramming that enables glutathione-mediated Cd resistance is the sulfur sparing response (Fauchon *et al*., 2002). This response to Cd involves a decrease in the production of abundant sulfur-rich proteins, allowing a greater proportion of cellular sulfur to be directed towards glutathione biosynthesis. Certain aspects of the above consequences of cadmium exposure overlap with responses reported for other particular metals or prooxidants (Lee *et al*., 1999 and Thorsen *et al*., 2007). Such overlaps in the stress response pathways of cells are common, whereas other effects appear to be restricted to particular subclasses of stressors. Thus, in studying the effects of redox-active agents (H_2O_2, copper and chromium), we and others have shown that a discrete subset of glycolytic enzymes within the cell become oxidized (Cabiscol *et al*., 2000; Costa *et al*., 2002; Shanmuganathan *et al*., 2004 and Sumner *et al*., 2005). It has been suggested, but not shown, that such a targeted oxidation of glycolytically associated enzymes could redirect the flow of carbon equivalents away from glycolysis and into the pentose phosphate pathway (PPP) (Costa *et al*., 2002; Shanmuganathan *et al*., 2004; Ravichandran *et al*., 1994 and Shenton and Grant, 2003), giving rise to elevated levels of cellular reducing power in the form of NADPH. In turn, it has been proposed that this could bolster cellular defenses against the immediate effects of redox-active stressors by, among other things, recycling cellular antioxidants such as reduced glutathione. Given the relatively rapid rate of targeted protein oxidation (within minutes) observed following exposure to redox-active metals (Shanmuganathan *et al*., 2004 and Sumner *et al*., 2005), if the association between protein

oxidation and cellular reducing power holds true, there could be a similarly rapid elevation in the levels of the necessary reducing power, NADPH. As described above, a large component of the extensive transcriptional reprogramming that occurs in Cd-stressed cells is directed towards increasing the availability of glutathione. With this in mind, here we hypothesize that the availability of any further mechanisms for increasing glutathione capacity (such as the possible reshuffling of carbon equivalents through the PPP) will have been assimilated into the cadmium stress response during evolution. Although a targeted PPP response has only previously been suggested for redox-active metals (see above), and other key responses to chemically disparate metals can be distinct (Lee *et al*., 1999; Gross *et al*., 2000; van Bakel *et al*., 2000 and Holland *et al*., 2007), here we show that redox-inactive cadmium elicits a pattern of protein oxidation in the cell comparable to that observed previously for copper and chromium (Shanmuganathan *et al*., 2004 and Sumner *et al*., 2005). Moreover, we show that this focused protein oxidation comprises part of a rapid and coordinated response that is specific to cadmium. This response serves to enhance reduced-glutathione availability and, consequently, Cd resistance.

Materials and Methods

Strains, media and metal exposure

Saccharomyces cerevisiae BY4741 (*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the isogenic mutants *zwf1*Δ and *gsh1*Δ (obtained from Euroscarf, Frankfurt, Germany) were routinely maintained on YEPD agar. Experimental cultures in YEPD broth were inoculated from 24-h starter cultures derived from single colonies and grown overnight to exponential phase (OD_{600} ~2.0) at 30 °C with orbital shaking (120 rev min^{-1}). At the start of each experiment,

cadmium nitrate [Cd $(NO_3)_2$] was added to flasks to a final concentration of 30 μM. The cultures were incubated with shaking, as defined above. Growth was monitored by optical density measurements at 600 nm.

Preparation of protein extracts

Yeast cells were grown to late-exponential phase, exposed to Cd $(NO_3)_2$ and harvested, as described above. Harvested cells were flash frozen and stored at -80ºC. Frozen cell pellets were resuspended in lysis buffer [100 mM Tris, pH7.4, 10% (v/v) glycerol, 1 mM PMSF, 264 mg ml^{-1} aprotinin, 20 mg leupeptin, 10 mg ml^{-1} pepstatin] and cells were disrupted with glass beads (0.5 mm diameter) using a mini-bead-beater (Biospec Products), interspersed with cooling on ice. Cell debris was removed by centrifugation (12,000 x *g*, 10 min, 4 °C) and supernatants retained for protein analyses. Protein concentration in the supernatants was determined according to Bradford or by using the BCA protein assay kit (Pierce Chemicals).

Two dimensional gel electrophoresis and Western blot analysis

2D-electrophoresis and Western blot analysis were performed as described earlier (Shanmuganathan *et al*., 2004) with one modification. For protein abundance detection, 40 μg of protein was derivatized with the florescent minimal dyes, Cy3 (control) and Cy5 (metal exposed sample), as defined by manufacturer (GE Life Sciences). The proteins were mixed and separated by 2D electrophoresis. For Western blotting to detect oxidized proteins, 100 μg of protein was used. All separations were carried out on pH 3-10 IPG strips and iso-electric focusing was performed according to the protocol of Gorg *et al.,* (2000). Strips containing samples intended for Western blotting were derivatized with 2,4-dinitrophenylhydrazine (DNPH) for 20 min

(Conrad *et al*., 2000) and washed in 2M Tris / 30% glycerol for a further 20 min. All protein-laden strips were then equilibrated in SDS buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol for 15 min, and containing 4% iodoacetamide for an additional 15 min. Proteins within the strips were resolved further by electrophoresis through 12.5% homogeneous polyacrylamide gels. Further separation in the second dimension was done through 12.5% homogeneous polyacrylamide gels. For analysis of carbonylated proteins by Western blotting, the derivatized proteins were electroblotted onto PVDF membranes. and probed with rabbit anti-DNP as primary antibody (Molecular Probes Inc; 1:16,000 dilution) and peroxidase-linked goat anti rabbit IgG as secondary antibody (Sigma; 1:16,000 dilution) (Conrad *et al*., 2000). Carbonylated proteins were immunodetected with a chemiluminescent peroxidase substrate, West femtoM (Pierce) and visualized using a Fuji LAS1000 Image Analyzer with pre-cooled CCD camera. For protein abundance detection, CyDye fluorescence was captured on a Typhoon scanner and the images were analysed using Decyder DIA software (GE Lifesciences).

Identification of proteins

For preliminary determination of proteins after 2D-resolution, images from the SYPROruby™ stained gels were compared to the 2D yeast proteome database (http://www.ibgc.u-bordeaux2.fr/YPM/) and the Swiss-2D-PAGE database (http://www.expasy.org/images/swiss-2dpage/publi/yeast-high.gif). The identity of specific proteins that were shown to be susceptible to carbonylation was confirmed by sequence analysis. Proteins of interest were excised from 2D gels that had been stained with 0.05 (w/v) Coomassie blue in 0.5% (v/v) acetic acid, 20% (v/v) methanol and subsequently destained with 30% (v/v)

methanol. The excised proteins were digested overnight with trypsin (11 ng/µl), and the masses of the resulting peptides analyzed by MALDI-mass spectrometry (Voyager DE Pro; Applied Biosystems). Identification of each sequence was based on sequences available in the SWISS-PROT protein database.

Real time RT-PCR

Transcript levels were measured by relative quantification real-time RT-PCR using a one-step Taq-Man RT-PCR kit and the 7500 SDS system (Applied Biosystems). Gene specific primers for ZWF1 (forward primer CGTCAGGTGATCTGGCAAAGA; reverse primer ACTTCAGGTCCTCCTCCATGG; and FAM-TAMRA dual labelled probe 6FAM-CCATCTACCAAGA-TCTTCGGTTATGCC-TAMRA) and gene specific primers for ACT1 (forward primer ATGCAAACCGCTGCTCAA; reverse primer AGTTTGGTCAATACCGGCAGA; and FAM-TAMRA dual labelled probe 6FAM-TGGTAACGAAAGATTCAGAGCCC-TAMRA) were designed using Primer Express software v2.0 (Applied Biosystems). After DNase treatment, 200 ng of total RNA with 1 µM primers and 0.2µM probes were used for RT-PCR under the following conditions: reverse transcription (30 min, 48°C), one cycle of denaturation (10 min, 95°C), and 45 cycles of PCR reaction comprising denaturation (15 s, 95°C) and annealing plus elongation (1 min, 55°C). Fold changes in gene expression were calculated using SDS software through the ΔΔCT method (Chung, 2001; Kapitanovic *et al*., 2006).

NADPH / NADP$^+$ assays

Wild-type yeast cells were exposed to 30 μM $Cd(NO_3)_2$ and cell extracts were prepared at 0, 5, 15, 30 and 60 min after Cd treatment, as described (Reekmans *et al*., 2005). Protein concentration was determined using the BCA protein assay kit (Pierce Chemicals). NADPH and $NADP^+$ concentrations were measured as described (Zhang et al., 2000). NADPH and $NADP^+$ concentrations were determined from standard curves and normalized to protein concentrations, before calculating the ratio of NADPH to $NADP^+$.

Glutathione assays

Extracts prepared from wild-type and *zwf1*Δ mutant cells were exposed to either 30 μM $Cd(NO_3)_2$ or 8mM $Cu(NO_3)_2$, with cell extracts being prepared at 0, 5, 15, 30 and 60 min after metal treatment, as described above, with protein concentration determined using a BCA protein assay kit from Pierce Chemicals. Reduced glutathione (GSH) was measured using Quantichrom glutathione assay kit (Bioassay Systems) according to manufacturer's instructions, with the amounts of GSH being normalized to protein concentrations.

Enzyme assays

Alcohol dehydrogenase (ADH) activity was measured in cell extracts according to the method of Bergmeyer (Bergmeyer, 1983). The assay buffer consisted of 100 mM Tris-HCl (pH 8.3), 2 mM NAD^+ and 0.8 M ethanol. The reaction was started by the addition of ethanol and the increase in A_{340} was monitored. Glyceraldehyde-3-phosphate dehydrogenase (TDH) activity was measured according to the method of McAlister and Holland (1985). The assay buffer was made up of 0.1 M potassium phosphate (pH 7.4), 1 mM NAD^+, 10 mM EDTA, and 0.1 mM DTT. The

reaction was started by the addition of 2 mM glyceraldehyde-3-phosphate and the increase in A_{340} was monitored. Enolase activity was measured according to the protocol of Poyner *et al* (1996) with one modification. The required substrate 2-PGA was generated from 3-PGA using phosphoglycerate mutase, which was included in the assay buffer. The assay buffer consisted of 30 mM Tris-HCl (pH 7.4), 2 mM magnesium acetate, 100 mM KCl, 0.15 mM NADH, 0.7 mM ADP, 14U pyruvate kinase, 20 U lactate dehydrogenase, 6.6 mM 3-PGA, 1.3 mM 2,3 DPGA, 0.2 U phosphoglycerate mutase. The decrease in A_{340} was monitored. Zwf1p activity was measured according to the method of Kudy and Noltman (Yue et al., 1969). The assay buffer was made up of 0.1 M phosphate buffer, pH 7.0, 1 mM $NADP^+$ and the reaction was initiated by the addition of 10 mM glucose 6-phosphate. The increase in A_{340} was monitored. Enzyme activities were measured in triplicate independently and normalized to the amount of total protein in each cell extract. Specific enzyme activities from the various cadmium-treated samples were normalized to their respective control samples.

Cadmium toxicity assays

Cells were grown in YEPD to an OD_{600} =1.0. Subsequently, aliquots were removed, serially diluted, and plated onto YEPD agar containing 30 μM $Cd(NO_3)_2$. Colony forming ability was determined after 3 - 4 days growth at 30^{o}C.

Results

Cadmium causes oxidation of glycolytic proteins

We tested the effects of cadmium exposure on protein oxidation in yeast cells using a sub-lethal $Cd(NO_3)_2$ concentration that produced a mild (about 17 min) increase in the cell doubling time, comparable to that established previously in a study of copper-induced protein oxidation (Shanmuganathan *et al*., 2004). Protein oxidation typically peaks within <60 min of exposure to redox active stressors (Shanmuganathan *et al*., 2004; Sumner *et al*., 2005 and Avery *et al*., 2000), so protein extracts were prepared from cells over this initial 1 h time course of incubation with Cd. Extracted proteins were derivatized with 2- dinitrophenylhydrazine (2-DNPH), which binds specifically to oxidized (carbonylated) proteins. Derivatized proteins in the pI range of 3-10 were then identified, by immunochemiluminescent labelling, and quantified. It was found that total protein oxidation increased by three-fold in as little as 5 min after Cd exposure (Figure 14). By 15 min, protein oxidation had peaked at levels 10- fold higher than in control samples, before stabilizing at a level that remained 4-fold elevated after 60 min. To identify specific protein-oxidation targets, cellular proteins were resolved with two-dimensional protein electrophoresis, and oxidized proteins were immunodetected. The cellular levels of proteins were similar in the untreated and Cd-treated cell cultures (Figure 15A, C). However, protein oxidation was increased in the Cd-treated cells (Figure 15B, D). Note that in preliminary control experiments carried out in the absence of Cd, neither protein levels nor oxidation profiles of proteins changed significantly over the time course of the experiment. Specific targets of Cd-induced protein oxidation that we identified are listed in Table 4. With the exception of actin (Act1), S-adenosylmethionine synthetase (Sam1) and some heat-shock protein chaperones, such as Hsp60, most of the identified oxidized proteins are known to exist in the cytosol as three or

more distinct isoforms. The majority of these proteins are involved either in glycolysis or in subsequent reactions of glucose catabolism: Adh1 (alcohol dehydrogenase), Eno1 (enolase I), Eno2 (enolase II), Fba1 (fructosebiphosphate aldolase), Pdc1 (pyruvate decarboxylase I), Pgk1 (phosphoglycerate kinase I), Tdh3 (glyceraldehyde-3-phosphate dehydrogenase). Subsequent to their identification, the degree to which each protein was oxidized after the initial Cd exposure was quantified and profiled over time (Figure 16). Protein oxidation was estimated densitometrically and normalized relative to protein abundance in the cell (Shanmuganathan *et al*., 2004 and Sumner *et al*., 2005). Act1, Sam1 and Eno1 exhibited the highest levels of oxidation. Generally, the times at which oxidation peaked in individual proteins mirrored that seen with total protein oxidation, although oxidation appeared to be more rapid in Sam1p and slower in Pdc1p (Figure 16). The oxidation levels in particular proteins did not always correlate with the proteins' relative abundances, even for isoforms of the same protein (Figure 15E-J). For example, oxidation levels of the different Pcd1p isoforms were quite distinct from their respective levels of abundance after 30 min of Cd exposure (Figure 15G-J). In addition to such apparently specialized oxidation profiles, there were also some specific, cadmium-dependent increases in the presence of key enzymes of glutathione and sulfur metabolism pathways, such as Cys3, Cys4 and Eno1 (Dormer *et al*., 2000) (Figure 15K, L). While such changes were not necessarily directly associated with any observed oxidation, the relative increases in the cellular concentrations of these proteins are consistent with the Cd-associated, sulfursparing responses previously reported (Fauchon *et al*., 2002).

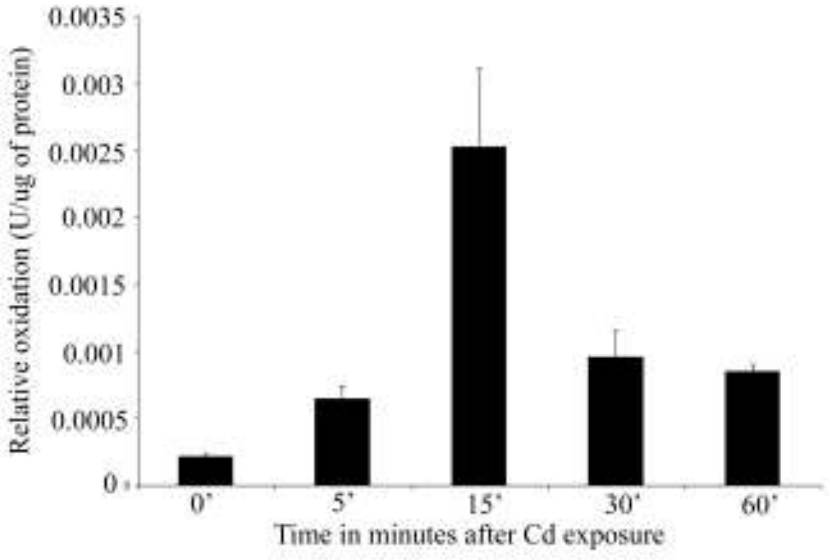

Figure 14. Cadmium induced protein oxidation in yeast.

Yeast cells were allowed to grow to an OD_{600} ~2.0 before being treated with 30μM Cd $(NO_3)_2$ for different time periods. Protein samples extracted from these cells were derivatized with 2-DNPH and oxidation was measured by immunochemiluminescent detection of DNP-bound proteins. Oxidation levels were normalized to protein expression levels and expressed as units / μg of protein. Values are representative of two gel separations and are shown here with their standard deviations.

Table 4. A list of all proteins that are oxidized within 60 minutes of 30μM Cd exposure

No.	Protein	Protein name	≈MW (KDa)	≈ pI
1	Act1	Actin	41.7	5.19
2	Adh1	Alcohol dehydrogenase I	36.8	6.06
3	Eno1	Enolase I	46.5	4.89
4	Eno2	Enolase II	46.8	5.56
5	Fba1	Fructosebiphosphate aldolase I	39.5	5.39
6	Hsp60	Heat shock protein 60	65.2	4.89
7	Pdc1	Pyruvate decarboxylase I	61.4	5.62
8	Pgk1	Phosphoglycerate kinase I	61.2	5.89
9	Tdh3	Glyceraldehyde-3-phosphate dehydrogenase III	35.6	4.79
10	Sam1	S-adenosylmethionine synthetase	41.8	4.91
11	Ssa1	Heat shock protein 70	73.2	4.89

Proteins were extracted from yeast cells before and upto one hour of 30μM Cd exposure. Extracts were separated by two-dimensional electrophoresis and oxidized proteins were identified using anti-DNPH antibodies and mass-spectrometry as described in materials and methods.

Cadmium decreases the activities of oxidized glycolytic proteins and increases the expression and activity of glucose-6-phosphate dehydrogenase (ZWF1)

To interpret any metabolic consequences of the Cd-induced, targeted protein oxidation described above, the enzymatic activities of some key glycolytic (glyceraldehyde-3-phosphate dehydrogenase [Tdh] and enolase [Eno]) and post-glycolytic (alcohol dehydrogenase [Adh]) proteins were measured in cell extracts from control and Cd treated cells. All three of these enzyme activities were decreased as a result of Cd exposure. After 30 min treatment with Cd, Adh, Tdh and Eno activity were 28%, 19% and 45%, lower than in control cells (Table 5), reaffirming our prediction that Cd-induced oxidation of these proteins would be associated with decreases in their activities.

It has been proposed elsewhere that a reduced rate of glycolysis, suggested by data such as ours (above), could result in a net increase in throughput of carbon equivalents through the pentose phosphate pathway (PPP) (Ravichandran *et al*., 1994; Shanmuganathan *et al*., 2004; Shenton and Grant, 2003). We hypothesized that cells in this scenario might upregulate PPP activity to accommodate the enhanced metabolic flow. To test this hypothesis directly, we examined the expression and activity of the first and rate-limiting enzyme of the PPP pathway, glucose-6-phosphate dehydrogenase, which is encoded by *ZWF1* (Kletzien et al., 1994; Thomas et al., 1991). *ZWF1* expression was monitored with real time RT-PCR and was found to increase more than 25-fold during the first hour of Cd exposure (Figure 17, inset).

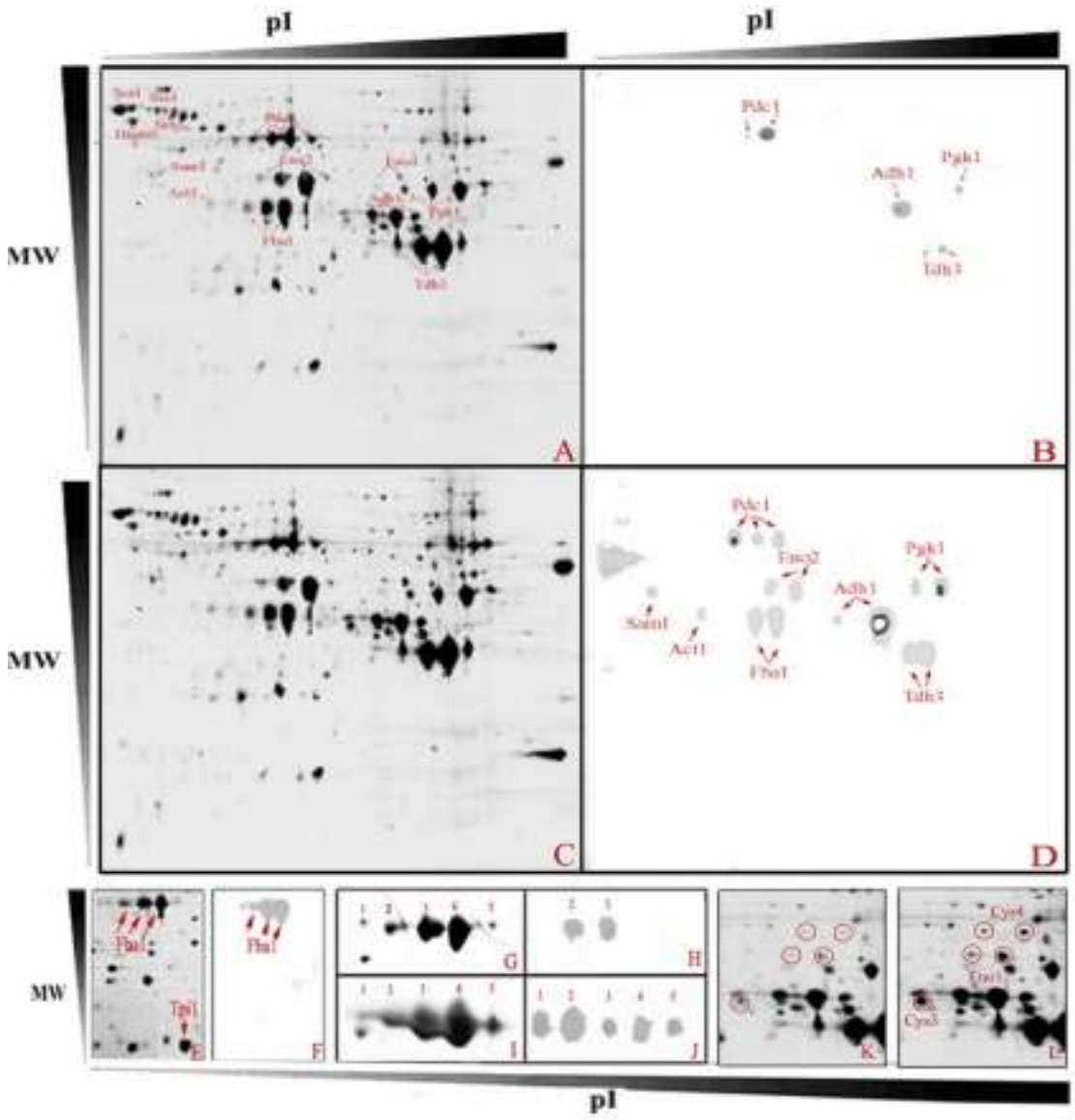

Figure 15. Specific targeting of cadmium-induced protein oxidation and expression profiles in yeast.

Proteins were extracted from yeast cells before and during various times of exposure to 30 μM Cd $(NO_3)_2$. Abundance profiles (Panels A, C, E, G, I, K and L) were obtained by derivatizing the proteins with Cy3 (control) and Cy5 (metal exposed) and then performing 2D electrophoresis. Western blots (B, D, F, H and J) were probed with anti-DNP antibodies for oxidized proteins. Panels A–D show abundances and oxidation profiles of proteins from cells before (A, B) and after (C, D) 60 min exposure to Cd. Panels E and F show the abundance and oxidation profiles of Fba1p and Tpi1p from cells exposed to Cd for 15 min. Panels G–J show the abundance and oxidation profiles of Pdc1p at 0 min (G, H) and 30 min (I, J) after Cd exposure. Panels K and L depict the expression profiles of Cys3, Cys4 and Eno1 before and after 60 min. of exposure to Cd respectively. Identified proteins are indicated by their standard abbreviations. Gels and blots are representative of replicate 2D separations undertaken after different times of Cd exposure.

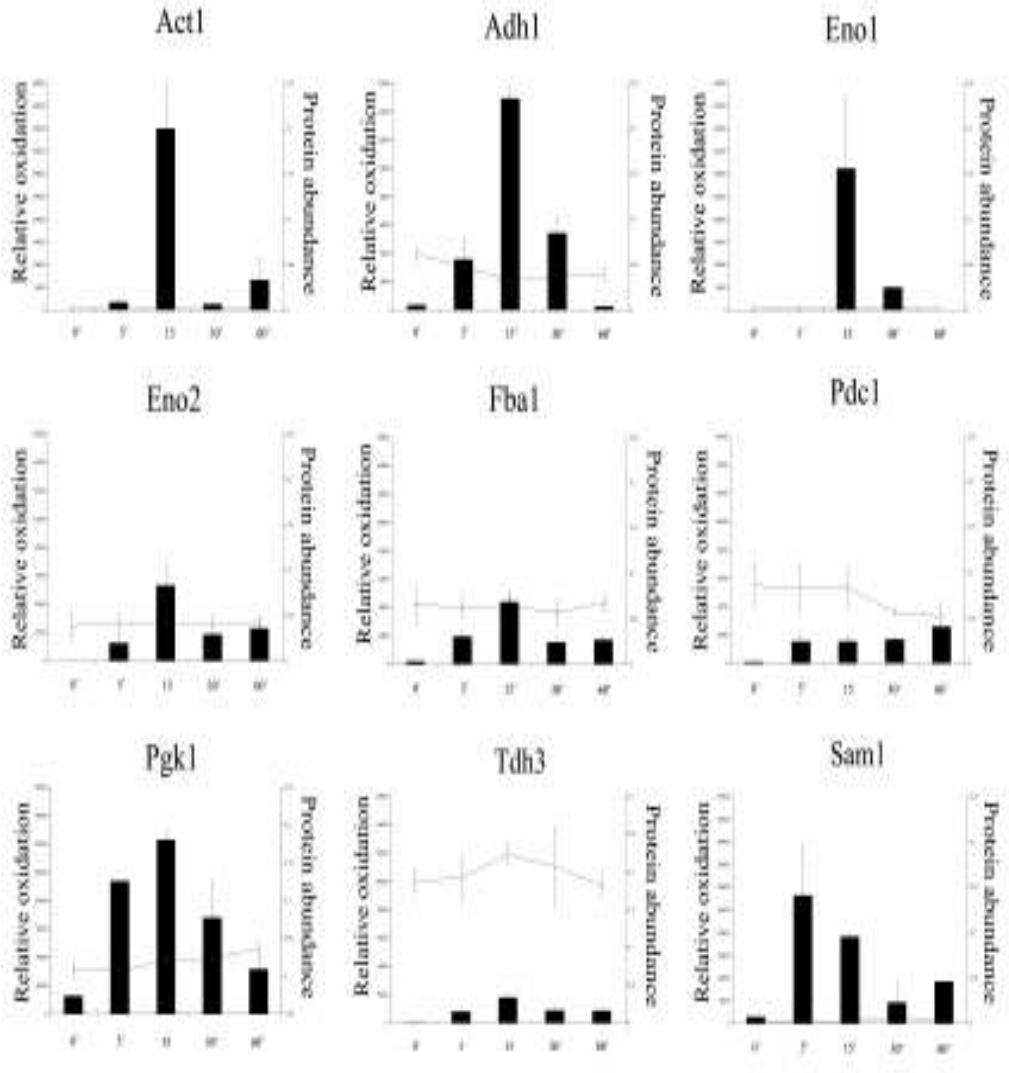

Figure 16. Profiles of individual proteins oxidized in response to cadmium.

Individual protein oxidation was quantified using the Fuji-Multigauge software from 2D-Western blots and normalized to protein abundance. Relative oxidation is shown as units of oxidation / μg of protein expressed. Protein abundance is shown as amount of protein present as a fraction of total protein. Values are representative of two gel separations and are shown here with their standard deviations.

Table 5. Inhibition in enzyme activity after 30 minutes of Cd exposure

	Protein	Protein name	% inhibition
1	Adh	Alcohol dehydrogenase	28
2	Tdh	Glyceraldehyde-3-phosphate dehydrogenase	19
3	Eno	Enolase	45

Protein extracts were prepared from yeast cells before and after 30 minutes of 30μM cadmium exposure. Activities of the individual proteins were measured spectrophotometrically in these extracts as defined in materials and methods. Enzyme activity was normalized to protein concentration and indicated here as percent inhibition in activity due to Cd exposure as compared to control.

Effects on Zwf1p activity levels were more modest but, nonetheless, Zwf1p activity did increase steadily during the 1 h exposure to Cd (Figure 17). These findings are consistent with a response to Cd that increases carbon flow through the PPP.

Cadmium increases the levels of NADPH and the ratio of NADPH to NADP$^+$

The PPP is known to be the major source of cellular reducing power (in the form of NADPH) in cells that use glucose as their primary carbon source (Minard et al., 1998; Slekar et al., 1996). The cellular NADPH/NADP$^+$ ratio is, therefore, considered to be a good indicator of carbon flow through the PPP, and a useful means to understand the potential metabolic changes in the pathway (Shenton and Grant, 2003). Having observed an increase in Zwf1p activity of Cd-treated cells, we measured the concentrations of NADPH and NADP$^+$ pools in cell extracts of treated and untreated cells (Zhang et al., 2000). The levels of NADPH were effectively unchanged up to 15 min after Cd exposure, but then increased rapidly by up to 3 fold (Figure 18A). Similarly, the ratio of NADPH to NADP$^+$ remained relatively constant during the first 15 min of Cd exposure, but had increased by ~7-fold after 60 min (Figure 18B), indicating that a significant portion of the enhanced reducing power in the cell (NADPH) is due to the reduction of NADP$^+$ into NADPH. To substantiate further that this Cd- induced increase in the NADPH to NADP$^+$ ratio was mediated by the pentose phosphate pathway, NADPH and NADP$^+$ levels were also measured in a PPP-defective *zwf1*Δ mutant. The NADPH/NADP$^+$ ratio was barely affected by Cd treatment in *zwf1*Δ cells (Figure 18C), indicating that the response observed in the wild type was PPP-dependent. This conclusion was confirmed with an alternative PPP mutant, *gnd1*Δ (result not shown).

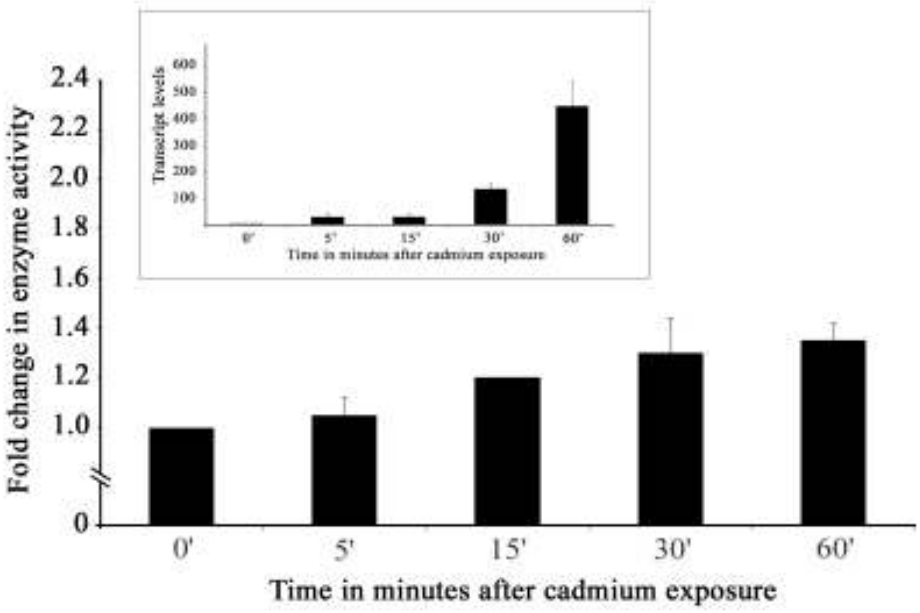

Figure 17. G-6P dehydrogenase enzyme (*ZWF*1) activity and *ZWF*1 gene expression in response to cadmium exposure.

Zwf1 activity was spectrophotometrically measured as defined in materials and methods. Transcript levels of *zwf1* were measured by relative quantification real time RT-PCR with β-actin as the endogenous control. The relative enzyme activity in cells exposed to cadmium is profiled over time within the first hour of exposure. Transcriptional expression of *zwf1* is similarly profiled over the same time course after exposure to cadmium (inset). Data shown are representative of two independent assays.

Cadmium induces a PPP-dependent increase in reduced glutathione (GSH) levels

Glutathione is an anti-oxidant tripeptide, which exists in either a reduced (GSH) or an oxidized form (GSSG). Re-reduction of the oxidized form is catalyzed by glutathione reductase, which uses NADPH as the source of reducing power (Holmgren, 1989). Because the PPP provides the major source of NADPH in cells, intracellular GSH pools are maintained primarily by the activity of two PPP enzymes, namely glucose-6-phosphate dehydrogenase and to a lesser extent 6-phosphogluconate dehydrogenase (Salvemini *et al*., 1999). Having observed an increase in the ratio of NADPH to $NADP^+$ (Figure 18B), we also measured reduced glutathione levels to ascertain if these reflected the increased NADPH / $NADP^+$ ratio. Cadmium exposure resulted in a >3-fold increase in the cellular concentration of GSH (Figure 19A). This result was consistent with the re-direction of carbon equivalents to the PPP in response to Cd, generating increased NADPH for GSSG reduction (see above). The involvement of the PPP in causing increased GSH levels was corroborated by a marked suppression of this response to Cd in a *zwf1*Δ mutant (Figure 19A). Thus, maintenance of cellular GSH was reliant on (albeit not exclusively) cellular glucose-6-phosphate dehydrogenase activity. Such changes in GSH levels appear to be a particular feature of a cellular response to cadmium, and were not apparent in similar experiments undertaken with other metal stressors, such as copper (Figure 19A inset). Levels of oxidized glutathione (GSSG) were also analyzed and routinely measured < 5% of GSH (data not shown). While small fluctuations in GSSG were observed, these proved to be statistically insignificant in comparison to changes in reduced glutathione.

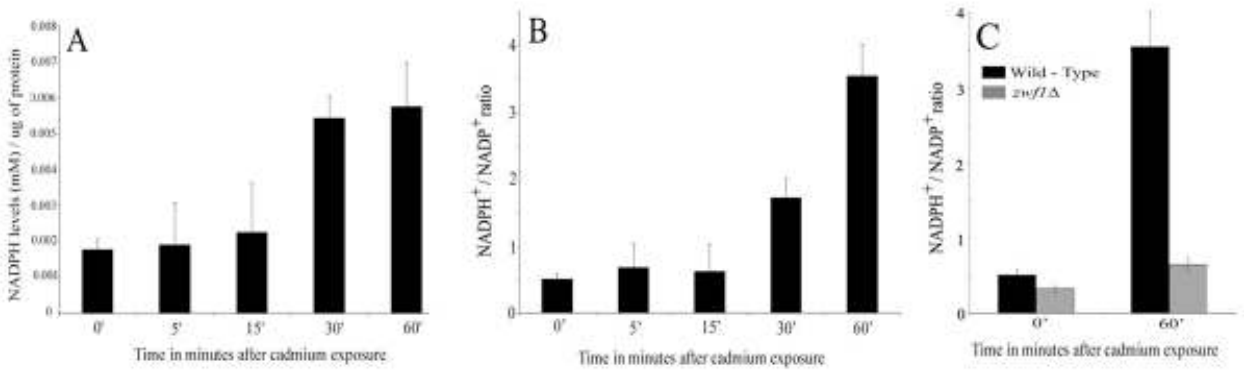

Figure 18. NADPH / $NADP^+$ ratios in response to cadmium exposure and Zwf1p-dependancy.

Nucleotide pyridines were spectrophotometrically measured as defined (Zhang et al., 2000) in extracts of untreated cells and those exposed to 30μM cadmium for various time periods. NADPH and $NADP^+$ values were normalized to protein concentrations of the extracts before calculating NADPH / $NADP^+$ ratios. NADPH levels (A) and NADPH / $NADP^+$ ratios (B) are shown. Data shown are representative of three independent assays.

As the GSH response to Cd was largely dependent on the PPP, it was hypothesized that the PPP is required for Cd resistance. The critical role for glutathione in Cd resistance *(*Li *et al*., 1997 and Smith *et al*., 2007*)* was confirmed here by Cd hyper-sensitivity in a *gsh1*Δ deletion strain (Figure 19B). z*wf1*Δ cells were also seen to be Cd sensitive, although to a significantly less extent in comparison to that of the *gsh1*Δ cells. This is in keeping with the fact that the reduction of glutathione was not eliminated altogether in the PPP mutant. Nonetheless, the results demonstrate that the PPP is required for optimal yeast growth in the presence of cadmium.

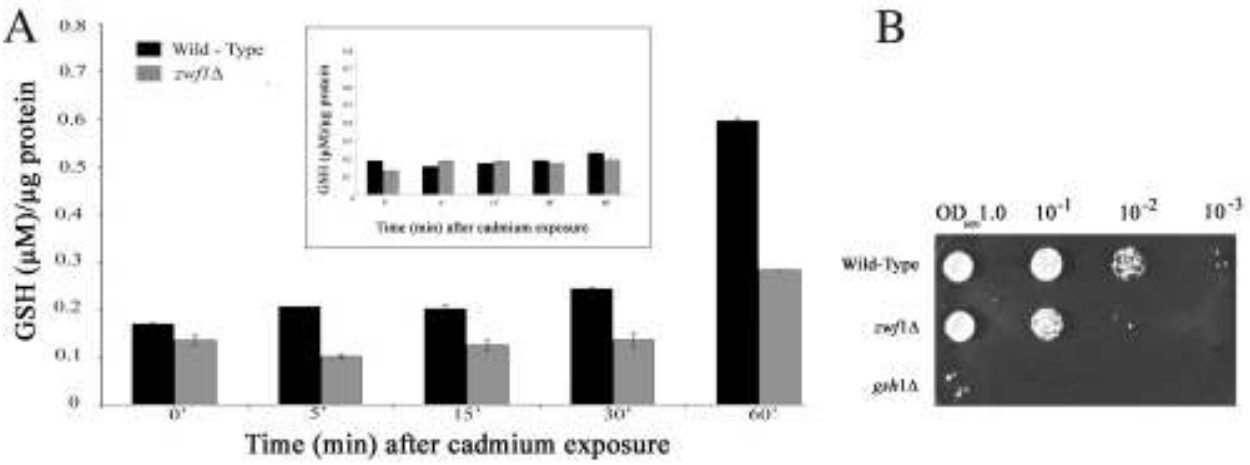

Figure 19. Zwf1p-dependency of Cd resistance and reduced-glutathione (GSH) levels during Cd exposure.

Cell extracts were prepared from wild-type and *zwf1*Δ yeast cells before and after exposure to 30 μM $Cd(NO_3)_2$, and GSH levels determined in cell extracts (see Materials and Methods). (A) Reduced GSH levels were normalized to protein concentrations of the extracts. The data shown are representative of three independent assays. Also shown (inset) are the corresponding levels of reduced GSH in cells exposed to 8 mM $Cu(NO_3)_2$ for a similar amount of time. (B) Serial dilutions of wild type, *zwf*1Δ and *gsh*1Δ cells growing in YEPD medium were spotted onto YEPD agar supplemented with 30 μM cadmium nitrate, before incubation for 3 d at 30°C. The growth of the strains was similar in the absence of cadmium (not shown).

Discussion

This paper reports that *S. cerevisiae* mounts a carbon-metabolism response to cadmium stress, which helps to support glutathione-dependent cadmium resistance. This adds a further facet to the glutathione responses that have already been reported in Cd-stressed cells, principally transcriptional induction of *GSH1* (Vido *et al*., 2001) and the sulfur-sparing response (Fauchon *et al*., 2002). The evident evolution of a multiplicity of congruent mechanisms to accomplish a similar outcome underscores the critical nature of glutathione for determining cell fate in the face of cadmium stress. The *GSH1* induction and sulfur-sparing responses described previously share the feature that they both promote the *de novo* synthesis of glutathione during Cd stress. This distinguishes the response highlighted here, which promotes the maintenance of pre-synthesized glutathione in (or its restoration to) a reduced state, accomplished by the re-direction of glucose equivalents from glycolysis to the NADPH-generating pentose phosphate pathway. The supply of reducing equivalents (NADPH) to glutathione reductase (Glr1p) is also critical for glutathione's detoxification role in response to oxidative stress (Grant, 2001), and elevated levels of Glr1p, in response to the presence of cadmium, were found to reflect this role (data not shown). Consequently, the coordinated cellular response described here appears to close the loop in the glutathione response story in that there is increased synthesis of glutathione, as well as a concomitant increase in synthesis of the reductant used by that enzyme to ensure sufficient levels of the functionally reduced form of this cellular thiol. The phenotypes of appropriate mutants show that each response is necessary to enhance glutathione-dependent Cd resistance. From an evolutionary perspective, it could also be predicted that the strengths of the various responses are likely to be coordinated, to maximize production of reduced-glutathione, while minimizing the creation of a bottleneck at one or more of the response pathways.

As well as upregulation of PPP activity, the carbon-metabolic response to Cd is coincident with decreased glycolytic enzyme activity. Selective oxidation (and denaturation) of glycolytic enzymes has been noted previously in response to redox-active stressors (Shanmuganathan *et al*., 2004; Sumner *et al*., 2005), but the suggestion that this could serve to enhance NADPH production for antioxidant defenses has not previously been tested directly. Temporal analysis in the present study showed that only a few proteins (primarily enzymes involved in either glucose metabolism or the fermentation of pyruvate) appear to be specifically targeted for oxidation. Although most of these oxidized proteins are also highly expressed in the cell, other proteins with similarly high expression, including Tpi1 (triose phosphate isomerase), were not oxidized (Figure 15E, F). Furthermore, the more abundant isoforms of certain enzymes (e.g., Pdc1) were oxidized to a lesser extent than their less abundant counterparts (Figure 15J). Such comparisons indicate that oxidation of these cellular proteins is a specific process, consistent with the proposal that this represents a targeted response. Such targeting may be a function of the isoforms of each enzyme being more susceptible to oxidation by virtue of its (their) configuration in the cell (Jolivalt *et al*., 2000; Shanmuganathan *et al*., 2004). Alternatively, the cellular location of each isoform may also contribute to its susceptibility. Proteomic analyses on mitochondrial proteins in yeast demonstrated that a number of glycolytically related enzymes are either attached to the mitochondrial membrane or present within the mitochondrion itself (Ohlmeier *et al*., 2004; Sickmann *et al*., 2003). As mitochondria are considered to be the primary oxidant source in the eukaryotic cell, the proximity or preferential association of these particular cytosolic enzymes to the outer membrane of the mitochondria may hold some clue as to why specific proteins and enzymes are more susceptible to oxidation than others. Whether it is the specific targeting of

particular isoforms or their preferential organellar association (or some combination of the two), the isoform-specific oxidation of these glycolytically related enzymes does provide a sensitive and rapid cellular reponse mechanism to conditions of oxidative stress. In this regard it is worth noting that all of the enzymes and proteins that were strongly oxidized contain one or more thiol groups (Le Moan *et al*., 2006), which are particularly susceptible to oxidation. Except for three of the enzymes (Eno1, Eno2 and Tdh3), none of these thiol groups are found in or near the active sites of their enzymes, which raises questions as to why these susceptible residues have been conserved in such an ancestral and highly evolved pathway of enzymes. It is tempting to speculate that the retention of such thiol groups may have preserved the very response that is proposed, i.e., a collective, transient oxidation of the glycolytic enzymes, which causes a coordinated, but temporary block in glycolysis in response to Cd stress (Shanmuganathan *et al*., 2004; Shenton and Grant, 2003).

In yeast, as in many other organisms that preferentially metabolize glucose, the PPP provides a metabolic alternative for the glycolytic intermediate, glucose-6-phosphate (G6P). The metabolic fate of G6P does not ultimately lie solely in the activity of subsequent rate-limiting enzymes within the glycolytic pathway; rather it lies in the activity of all the enzymes in the pathway, as well as the need for ATP (Fell, 1996; Hofmeyr, 1997; Kacser and Burns, 1995). Thus, the net effect of the focused, but transient, oxidation and inactivation of glycolytic enzymes observed here would be a synchronous burst of carbon equivalents through the PPP (Slekar et al., 1996). The first enzyme in the PPP, glucose-6-phosphate dehydrogenase (*ZWF*1) together with 6-phosphogluconate dehydrogenase (which catalyzes the third step in the pathway) produces the majority of NADPH in cells grown on glucose (Minard *et al*., 1998), and has been

shown to protect against peroxide-induced oxidative stress (Izawa *et al*., 1998; Minard and McAlister-Henn, 1999; Salvemini *et al*., 1999). The activity of Zwf1p has been used to determine flow through the PPP (Shenton and Grant, 2003) and so, for a similar purpose, we focused on Zwf1p in this study. Zwf1p activity steadily increased in response to Cd, a change that was surpassed by the 20-25 fold increase in *ZWF1* gene expression. Given the role of Zwf1p and its notably conservative response to dramatic metabolic shifts (Jeppsson et al., 2002; Thomas et al., 1991), the relatively modest increase in its enzyme activity (observed in Figure 1) demonstrates how tightly the activity of Zwf1p is maintained. This is presumably to ensure optimal flow of glucose-6-phosphate into the glycolytic pathway, and to avoid any potentially damaging redox imbalance within the cell. Even so, the increase in Zwf1p activity appears to be sufficient to accommodate for and sustain an increased carbon flow through the PPP, resulting in a marked Zwf1p-dependent increase in cellular NADPH levels. The coincident nature of the surge in levels NADPH with the maximal oxidation of the glycolytic enzymes 15 - 30 minutes after Cd exposure is consistent with a coordinate metabolic response of the cell to cadmium-induced oxidative stress.

As the major source of reducing power in the cell, activity of the PPP is closely tied to the cells' ability to regenerate the reduced form of glutathione (GSH), one of the more abundant cellular thiols (Salvemini *et al*., 1999). Glutathione protects cells directly from various ROS either by effectively scavenging free-radicals, or by acting as a co-factor for defense enzymes such as glutathione peroxidases (Avery, 2001; Wheeler and Grant, 2004). Glutathione is also able to chelate Cd directly, forming an S-conjugate that is then removed to vacuoles through the action of a cadmium inducible conjugate transporter (Li *et al*., 1997; Vido *et al*., 2001). GSH has

thus been shown to be an essential defense against Cd, as exemplified by the extreme Cd-sensitivity of a *gsh1Δ* deletion mutant (Figure 19B). The role of GSH in Cd resistance is predicated upon its reduction with available NADPH. Thus, the activity of Zwf1p and the PPP may also be reflected by GSH levels in the cell (Salvemini *et al*., 1999). The sensitivity of this relationship was demonstrated here by the Zwf1p-dependent >3-fold increase in reduced glutathione levels within the first hour of Cd exposure. This dependency on Zwf1p was not, however, absolute. Intracellular levels of reduced glutathione were only halved by the loss of *ZWF*1, with the concentration of reduced glutathione still increasing over time in response to cadmium, albeit at a slower rate. In the absence of *ZWF*1, other dehydrogenases such as acetaldehyde dehydrogenase (*ALD*6), isocitrate dehydrogenase (*IDP*2) and old yellow enzyme (*OYE*) can be recruited to fulfill, at least partially, the role of glucose-6-phosphate dehydrogenase under conditions of cellular and oxidative stress (Haarer and Amberg, 2004).

The decreased (but not abolished) GSH response in the *zwf1*Δ mutant was mirrored by the fact that *zwf1*Δ cells were more sensitive to Cd than wild-type cells, but more resistant than *gsh1*Δ cells. Therefore, the PPP-dependent response described in this paper is required to optimize glutathione mediated resistance to Cd stress. This represents a key new addition to the range of tools already known to be deployed by yeast to assure the effectiveness of GSH in countering Cd toxicity. The existence of such a range of responses, targeted specifically to supporting glutathione-mediated defenses, reinforces the critical nature of this thiol molecule for cell survival during stress, while also accentuating the metabolic ties that enable it to accomplish these tasks.

Acknowledgements

This research was supported by grants from the National Institute of Health (R01 GM57945), the Georgia Research Alliance, and a Molecular Basis of Disease fellowship at Georgia State University awarded to A. S.

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CHAPTER 4

Differential Protein Expression in *S. cerevisiae* in Response to Copper and Cadmium Exposure

Introduction

Copper is an essential trace element, which is structurally and catalytically incorporated into a number of critical metalloenzymes such as cytochrome C-oxidase, superoxide dismutase (Fernandes *et al*., 1998; Linder and Hazegh-Azam, 1996; O'Halloran and Culotta, 2000 and Stearman *et al*., 1996). These enzymes are involved in quite diverse cellular functions; including growth, respiration and antioxidant defense. In excess, however, copper is toxic and (as we have demonstrated) induces oxidative stress in yeast, even under limited exposure to low, effectively sub-lethal levels (Shanmuganathan, 2004). In higher organisms, copper toxicity is not only believed to be carcinogenic (Kawanishi *et al*., 2002), but is also known thought to be involved in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease (Becaria *et al*., 2003; Bush 2003 and Strausak *et al*., 2001) and autism (Wecker *et al*., 1985). In humans, an excess level of copper resulting from faulty copper excretion can manifest itself in the heritable condition, Wilson's disease and can ultimately result in liver, brain and kidney damage (Ala *et al*., 2007 and Gitlin, 2003).

While copper is essential to cellular function, cadmium is a "spurious contaminant of biological matter" (Kagi and Vallee, 1960) that has effectively no essential biological function, although it has been shown to play some beneficial role in the carbonic anhydrase of marine diatoms (Lane, 2000). The metal ion generally enters the ecosystem as a pollutant, primarily persisting in the ecological food-chains because of its long biological half-life (~30 years). Cadmium is extremely toxic even at low concentrations, and (like copper) is a potent carcinogen (Waalkes, 2003). Also, like copper, cadmium is associated with neurological impairments, exacerbating the effect of attention deficit hyperactivity disorders (ADHD) and autism (Deth *et al*., 2008 and Kern *et al*., 2007) as well as nephrotoxicity and kidney damage (Friberg, 1984).

Both metals are capable of inducing oxidative stress, albeit by different mechanisms (Chapter 1 and 3), and it is generally held that oxidative stress is, at least in part, the basis of metal toxicity of either metal (Avery and Avery, 2001; Avery, 2001; Cadenas, 1989; Jamieson, 1998 and Santaro and Thiele, 1997). Copper is redox-active and is capable of producing reactive oxygen species (ROS) (See General Introduction). As such, Cu can potentially mediate oxidative stress by directly reacting with the by-products of aerobic metabolism, giving rise to superoxide radicals. In contrast, cadmium has an entirely different redox chemistry and is incapable of producing ROS directly. Accordingly, it is considered to be redox-inactive and induces oxidative stress indirectly by displacing redox-active metals from enzymes (Srinivasan *et al*., 2000), potentially by depleting the antioxidant glutathione (Stohs and Bagchi, 1995) or through mitochondrial perturbation (Pacheco *et al*., 2007). The importance of such differences in the nature of the redox capacity of the two metals can be most clearly highlighted by the distinct transcriptional responses that are evident, in *Saccharomyces cerevisiae*, to different pro-oxidants,

such as H_2O_2, and the potentially more potent, superoxide generating, menadione (Fernandes *et al*., 2007; Gasch and Werner-Washburne, 2002 and Mutoh *et al*., 2005). Consequently, given the overt oxidative stress response in *Saccharomyces cerevisiae* that results from limited exposure to either copper or cadmium (discussed in the previous chapters), it is essential that we also study the concomitant changes in protein expression that may also result from exposure to either of these heavy metals.

In *Saccharomyces cerevisiae*, there are both overlaps and differences in the responses to these two metals. Thus, while Cd induces transcriptional factors yAP-1 and Met4, which in turn mediate Cd resistance (Fauchon *et al*., 2002; Wemmie *et al*., 1994 and Wu *et al*., 1993), Cu has not been shown to rely on yAP-1. Copper instead induces the transcription factor Ace1, which controls metallothionein synthesis (Cup1), as well as superoxide dismutase (Sod1), both of which mediate copper resistance (Culotta *et al*., 1989; Furst and Hamer, 1989 and Gralla *et al*., 1991). In contrast, *Saccharamyces cerevisiae* relies on the antioxidant glutathione for providing resistance to cadmium, and while glutathione is known to also complex with intracellular Cu ions (Freedman, 1989) the loss of glutathione production in a *gshA-2* mutant background had little effect upon cellular resistance to Cu (Gharieb and Gadd, 2004).

Pretreatment with H_2O_2 protects against Cd toxicity but not Cu toxicity. Also, while the antioxidant vitamin E has been shown to protect against Cd toxicity, it appears to have little affect upon Cu toxicity (Pourahmad and O'Brien, 2001). Pretreatment with aspirin has been demonstrated to offer some protection against both copper and cadmium toxicity (Mattie and Freedman, 2001). Stress responses in prokaryotes have also shown to differ between cadmium

and copper and the protein expression response to cadmium is thought to include a set of cadmium-specific proteins as well as some of the general stress proteins (Ferianc *et al*., 1998). The differences in the protective effects of vitamin E and aspirin on copper and cadmium toxicity may also indicate not only alternative mechanisms of metal toxicity, but also different types of stress responses. Studying the proteomic responses that are elicited by the two metals will help us more clearly define the global mode of cellular responses to either copper or cadmium, as well as to appreciate the overlaps and differences in their responses. This in turn will provide better insight into the mechanisms of toxicity mediated by these two metals.

Previous works on proteomic responses to pro-oxidants have analyzed changes that occur after prolonged exposure (Godon *et al*., 1998 and Vido *et al*, 2001). While the use of such a parameter has provided us with some understanding of the after-effects (or outcomes) of toxicity, these studies failed to delineate the immediate responses, which may ultimately decide the cellular fate. Moreover, genetic responses to various stressors have been shown to be transient and to reach steady-state levels in a short time frame (Gasch and Werner-Washburne, 2002). Studies in our laboratory have further demonstrated that protein oxidation levels are maximal within the first hour of exposure to metals (Chapters 1, 2 and 3). Mindful of these changes, we have monitored the protein response that occurs within the first hour of metal exposure, and have identified some of the more immediate proteomic responses to both copper and cadmium. Moreover, in order to ensure that the proteins under investigation are truly representative of all cells in the culture and are not varied as a function of live versus dead cells, we have employed sub-lethal concentrations of copper and cadmium in our analyses, as shown in previous chapters.

Materials and Methods

Strains, media and metal exposure

Saccharomyces cerevisiae BY4741 (*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was routinely maintained on YEPD agar. Experimental cultures in YEPD broth were inoculated from 24-h starter cultures derived from single colonies and grown overnight to exponential phase (OD_{600} ~2.0) at 30 °C with orbital shaking (120 rev min^{-1}) (Avery and Avery, 2001). At the start of experiments, copper nitrate $Cu(NO_3)_2$ or $Cd(NO_3)_2$ was added to flasks to a final concentration of 10 mM or 30 μM, respectively, and cultures were incubated with shaking as above. Growth was monitored with optical density at 600 nm. At intervals during metal exposure, cell samples were harvested by centrifugation and flash-frozen.

Preparation of protein extracts

Frozen cell pellets were resuspended in lysis buffer (100 mM Tris, pH 7.4, 10% (v/v) glycerol, 1 mM PMSF, 264 mg ml^{-1} aprotinin, 20 mg leupeptin, 10 mg ml^{-1} pepstatin). Cells were disrupted with glass beads (0.5 mm diameter) using a mini-bead-beater (Biospec Products), interspersed with cooling on ice. Cell debris was removed by centrifugation (12,000 x *g*, 10 min, 4 °C) and supernatants retained for protein analyses. Protein concentrations in the supernatants were determined according to Bradford (Bradford, 1976).

Protein labeling and Differential In-Gel Electrophoresis

CyDye DIGE flourescent minimal dye (GE Lifesciences) was reconstituted in dimethyl formamide according to manufacturer's instructions. 50 μg of protein sample from cells unexposed to metal (control) was derivatized with 200 pmol of Cy3 and 50μg of protein sample

from cells exposed to metal (sample) was derivatized with 200 pmol of Cy5. Derivatization with CyDyes was carried out for 30 minutes on ice and in the dark to achieve minimal labeling of protein samples. 10 mM lysine was added to the reaction mixture and incubated on ice for 10 minutes to stop the reaction. The labeled samples (Cy3 for untreated sample and Cy5 for metal treated sample) were then combined with re-hydration solution (8 M urea, 0.4% DTT, 4% CHAPS and 1% IPG buffer, pH- 3-10) and loaded onto pH 3-10 18 cm IPG strips. Iso-electric focusing was performed according to Gorg *et al* (2000). Before the second dimension, all strips were equilibrated in a SDS buffer (50 mM Tris-Cl, pH 8.8, 6M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol for the first 15 minutes and 4% iodoacetamide for the second 15 minutes. The strips were then electrophoresed on 12.5% homogenous polyacrylamide gels.

The gels were imaged with the Typhoon imager at appropriate wavelengths (GE Life Sciences) and analyzed using the Decyder DIA software (GE Life Sciences).

Identification of proteins

For preliminary determination of proteins after 2D resolution, images from the Cy-Dye gels were compared to the 2D yeast proteome database (*http://www.ibgc.u-bordeaux2.fr/YPM/)* and the Swiss-2D-PAGE database (*http://www.expasy.org/images/swiss-2dpage/publi/yeast-high.gif*; (Gorg *et al*., 2000). The identity of specific proteins within the protein array that were differentially expressed was confirmed by sequence analysis. Proteins of interest were excised from 2D gels that had been stained with 0.05 % (w/v) Coomassie blue in 0.5% (v/v) acetic acid, 20% (v/v) methanol, and subsequently destained with 30% (v/v) methanol. The excised proteins were digested overnight with trypsin (11 ng/µl), and the masses of the resulting peptides analyzed by MALDI-mass spectrometry (Voyager DE Pro; Applied Biosystems). Identification

of each sequence was based on sequences available in the SWISS-PROT protein database (Costa *et al*., 2002).

Results

Two-dimensional electrophoresis is a powerful technique that enables the simultaneous separation, identification and potential quantification of hundreds of proteins present in any given extract of the cell. However, since its inception as a useful analytical tool in the mid-1970's (O'Farrell, 1975), it has suffered from lack of reproducibility and considerable gel-to-gel variation. The use of Cy-Dyes, coupled with two-dimensional electrophoresis, has enabled the separation of different protein samples within the same gel. This has effectively removed many of the concerns, which involved gel-gel variation, and has dramatically increased reproducibility as well as significantly improved accuracy of quantification. Using this technique, we have separated the proteins of the yeast *Saccharomyces cerevisiae* and identified over 1,000 protein spots. We have analyzed changes in protein abundance that have taken place within an hour of exposure to sub-lethal levels of two metals, copper and cadmium, which are mechanistically different in their toxic effects upon the cell. Within as little as 15 minutes after metal exposure, a few discrete proteins were found to have changed in abundance. By 30 minutes, ~15 % of the detected proteome and by 60 minutes, up to 25% of the discernable proteins in the yeast proteome had changed in abundance (Figure 20, 21 and Table 6). For most of the proteins that exhibited variation in concentration in response to either copper or cadmium, gradual changes in their expression levels were detectable within 15 minutes of metal exposure, indicating the relative speed of the response. As indicated in Table 6, these differentially expressed proteins

belonged to various categories – metabolic enzymes, heat-shock proteins, anti-oxidant defenses, protein synthesis and amino acid biosynthesis. The relative proportion of proteins that are differentially expressed is small, when compared to earlier studies of proteomic response to H_2O_2 and cadmium (Godon *et al*., 1998 and Vido *et al*., 2001). This was not entirely unexpected, considering the relatively low, sub-lethal dose of each metal used, and the shorter periods of time (<60 minutes) after metal exposure at which samples were analyzed. Moreover, while a number of similarities are apparent between the results (shown in Table 6 and previous findings), a number of quite intriguing changes are noteworthy.

Differential Expression of Protein Translation Factors

In eukaryotes, protein synthesis is assisted by a number of translation factors, which play redundant roles in the process of protein synthesis. We found that a number of protein translation factors were repressed following both Cu and Cd exposure. Tif1 (eukaryotic translation initiation factor 4A) and Hyp2 (translation initiation factor 5A) were protein translation initiation factors that decreased by 2 fold and 3 fold respectively. Decreases in the levels of elongation factor 2A (Eft1) (Figure 22) and elongation factor 1 (Efb1) were also seen. Moreover, Rps5 and Rla0, the proteins that make up the 40S and 60S subunits of the ribosome, also decreased in response to cadmium exposure.

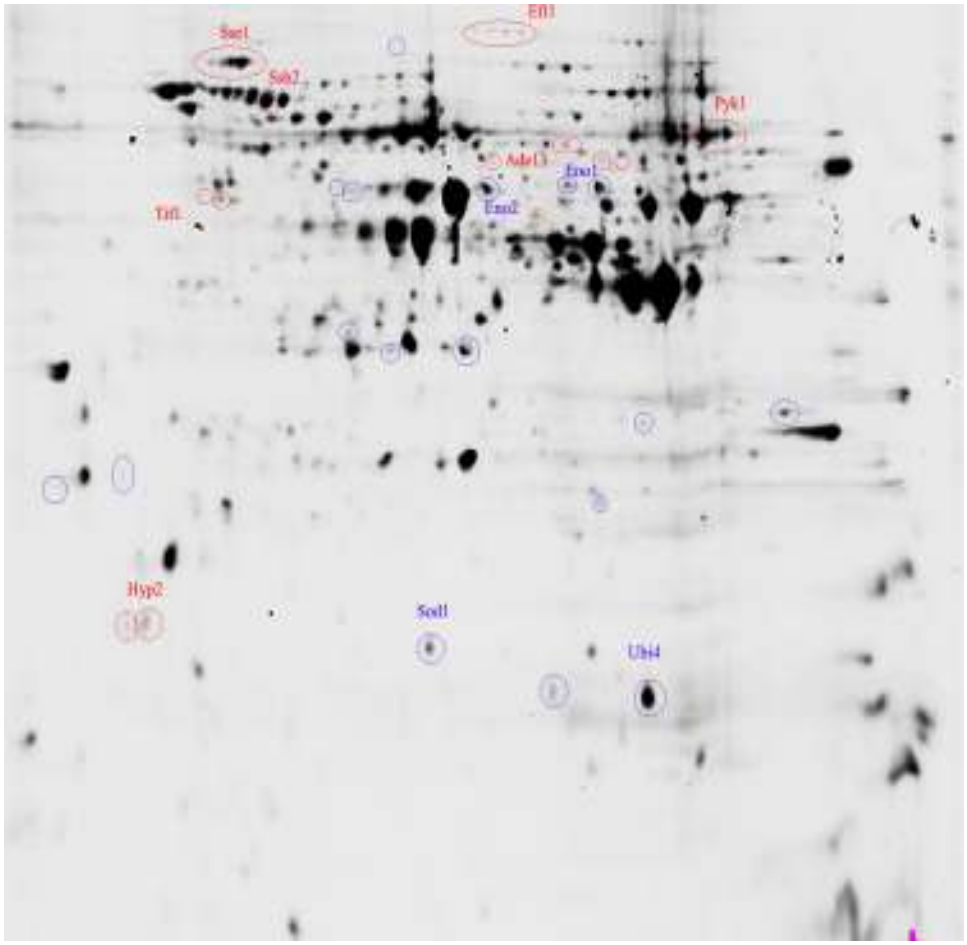

Figure 20. Differential expression of proteins seen after an hour of 10mM copper exposure

Proteins were extracted from yeast cells before and after an hour of 10mM $Cu(NO_3)_2$ exposure and labeled with Cy3 and Cy5 respectively. The labeled proteins were resolved by two-dimensional electrophoresis as described before. Differential expression was analyzed using the Decyder DIA software. Spots that are upregulated (>1.5 fold) after copper exposure are outlined in blue and those that are downregulated (<1.5 fold) are outlined in red.

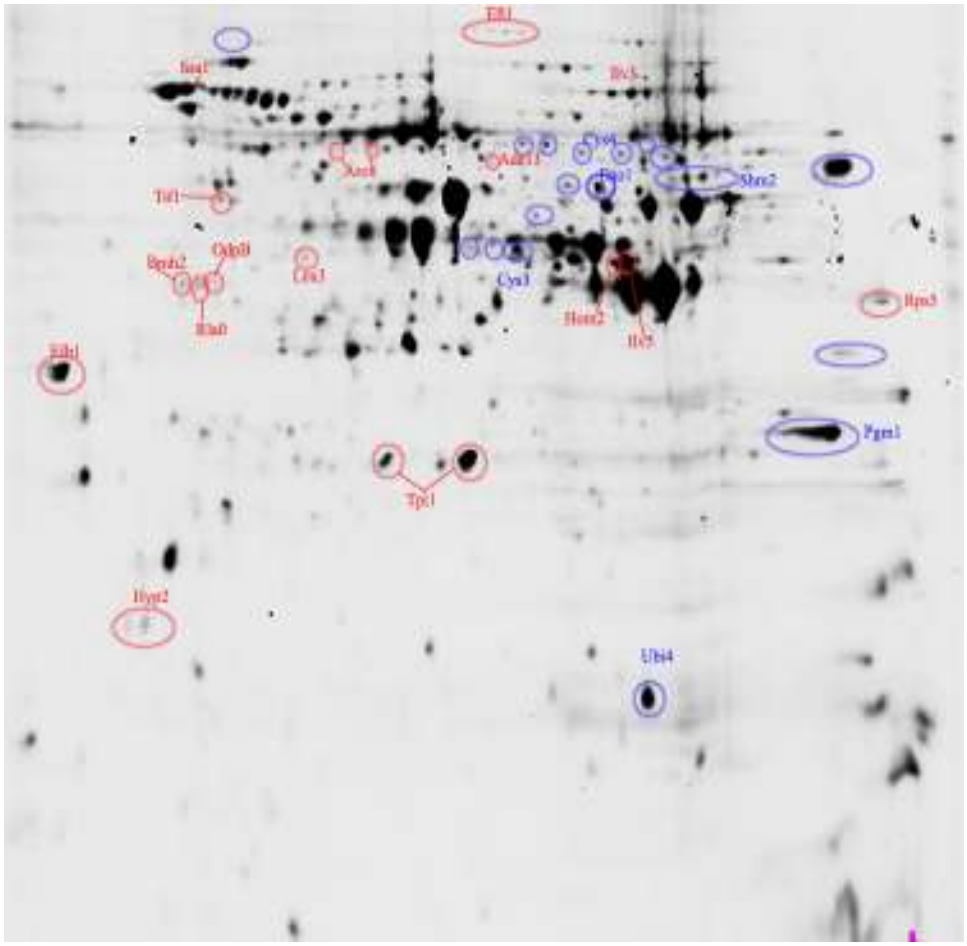

Figure 21. Differential expression of proteins seen after an hour of 30 μM cadmium exposure

Proteins were extracted from yeast cells before and after an hour of 30 μM $Cd(NO_3)_2$ exposure and labeled with Cy3 and Cy5 respectively. The labeled proteins were resolved by two-dimensional electrophoresis as described before. Differential expression was analyzed using the Decyder DIA software. Spots that are upregulated (>1.5 fold) after cadmium exposure are outlined in blue and those that are downregulated (<1.5 fold) are outlined in red.

Table 6. Proteins that are differentially expressed within 60 minutes of copper and cadmium exposure in the yeast *S. cerevisiae*

	Category	Std. Name	Protein Name	~MW (KD)	~pI	Fold change after 60' of Cu	Fold change after 60' of Cd
1	Protein synthesis & translational fidelity	Eft1	Elongation factor 2	98,000	6.2	-2.2	-1.5
		Hyp2	Initiation factor 5A	15,400	4.7	-2.9	-1.1
		Efb1	Elongation factor 1	30,000	4.3	Similar	-1.3
		Tif1	Translation initiation factor 4A	45,000	4.95	Similar	-1.5
		Rps5	Component of 40S ribosome	25,000	9	Similar	-2.3
		RlaO	Component of 60S ribosome	26,000	5	Similar	-1.6
2	Heat Shock proteins	Ssa1	Heat shock protein	70,000	4.82	+1.7	-2
		Sse1	Heat shock protein 82	80,000	4.97	-2.0	-1.7
		Hsp60	Heat shock protein 60	60,000	5.0	-1.8	-1.4
3	Metabolic enzymes	Eno1	Enolase 1	46,500	6.5	+2.3	+2
		Pdc1	Pyruvate decarboxylase	61,500	6.1	-1.3	-1.2
		Pgm1	Phosphoglycerate mutase 1	26,000	8.9	Similar	+1.7
		Tpi1	Triose phosphate isomerase 1	23,100	6	Similar	-1.4
4	Antioxidant Enzymes	Sod1	Cu-Zn superoxide dismutase	14,200	5.8	+2.3	Similar
		Ubi4	Ubiquitin	9,300	6.8	+3.1	+1.4
5	Amino acid Biosynthesis	Aro8	Aromatic aminotransferase I	56,200	5.9	Similar	-1.9
		Hom2	Aspartic beta semi-aldehyde dehydrogenase	39,400	6.66	Similar	-1.5
		Ilv5	Acetohydroxyacid reductoisomerase	39,500	6.7	Similar	-1.5
		Ilv3	Dihydroxyacid dehydratase	63,000	7.8	Similar	-1.8
6	Nucleotide metabolism	Ade13	Adenylosuccinate lyase	54,500	6.4	-2.7	-1.5
7	Sulfur metabolism	Cys4	Cystathionine beta-synthase	56,000	6.6	Similar	+2.4
		Cys3	Cystathionine gamma-lyase	42,500	6.52	Similar	+1.9

These results are in agreement with the findings of other groups (Gasch *et al*., 2002; Causton *et al*., 2001; Warner, 1999 and Jin *et al*., 2008). Protein synthesis is an energy expensive process and is almost always minimized during times of stress (Shenton and Grant, 2003; Shenton *et al*., 2006). It is, therefore, not too surprising that effects on protein synthesis and the protein synthetic machinery are apparent immediately, even after exposure to very low doses of metal, and are common to copper and cadmium insult. We also verified that the changes in the transcript levels of *eft1 and hyp2* reflected the changes in protein abundance, showing an underlying transcriptional basis to these changes.

Cadmium induces proteins involved in sulfur metabolism while copper does not

It is well established that the antioxidant tripeptide glutathione is an important player in Cd detoxification and, therefore, a large part of the cellular response to cadmium is geared to produce glutathione (Vido *et al*., 2001; Li *et al*., 1997 and Chapter 3). To serve this purpose, cadmium is known to induce the expression of the sulfur assimilation and glutathione production pathways (Vido *et al*., 2001) through the transcriptional activator, Met4 (Fauchon *et al*., 2002). Consistent with these previous findings, we observed a 2-3 fold induction in 2 enzymes of this machinery, namely Cys3 and Cys4 (Figure 23), which encode cystathionine beta synthase and cystathionine gamma lyase, two enzymes that catalyze the reactions synthesizing cysteine from homocysteine, the precursor for glutathione. Other Met4 responsive proteins, Met6 and Met25, that are involved in sulfur assimilation, while induced by higher concentrations of cadmium (Fauchon *et al*., 2002), remained unaltered in response to a 60 minute exposure to a sub-lethal dose of cadmium. Copper on the other hand, did not elicit any response

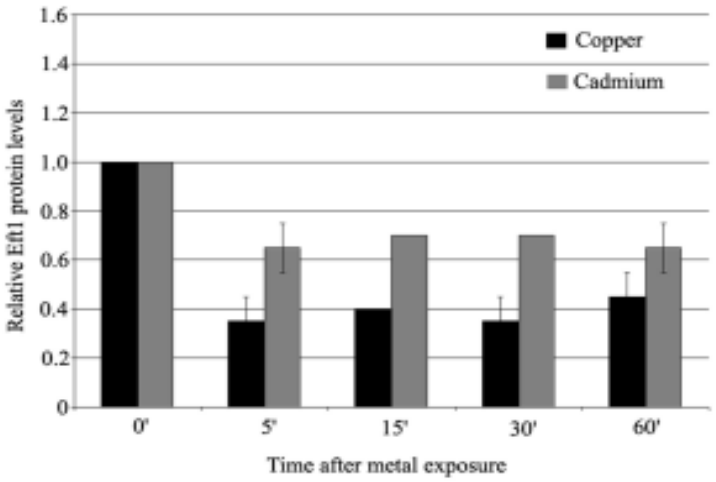

Figure 22. Eft1 decreases in response to both copper and cadmium exposure

Eft1 levels were analyzed by Cy-Dye DIGE before and after varying times of 10 mM copper or 30 μM cadmium exposure. Expression levels were normalized to control and expressed as relative units.

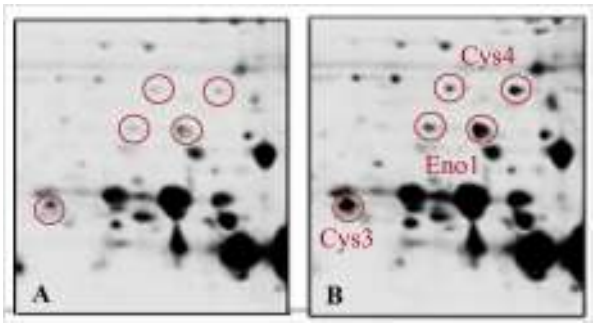

Figure 23. Cys3, Cys4 and Eno1 increase in response to cadmium exposure

Proteins were labeled with CyDyes before (A) and after an hour of 30 μM cadmium (B) exposure. The positions of the spots, Cys3, Cys4 and Eno1 are indicated by red circles.

from these proteins, consistent with the findings of Gharieb and Gadd, (Gharieb and Gadd, 2004), that copper resistance does not require a glutathione mediated response.

Differential expression of proteins involved in carbon metabolism

Glycolytic enzymes are also part of the proteomic response to copper and cadmium. Eno1 (enolase) is upregulated by 2-fold in response to both copper and cadmium (Figure 23 and Figure 24). Phosphoglycerate mutase (Pgm1) is increased only in response to cadmium (Figure 21), while pyruvate kinase (Pyk1) is only increased in response to copper (Figure 20). Other glycolytic proteins such as pyruvate decarboxylase (Pdc1), triose phosphate isomerase (Tpi1) and alcohol dehydrogenase (Adh1) are decreased following exposure to either metal. The other enzymes of glycolysis – Fba1 (Fructosebiphosphate aldolase), Eno2 (enolase), Pgk1 (phosphoglycerate kinase) and Hxk (Hexokinase) - do not show any remarkable changes in their expression levels within the first hour of exposure to either copper or cadmium. Thus, the enzymes of carbon metabolism do not all respond in a similar manner. It is conceivable that the decreases in levels of some proteins are compensated by an increase in some others so that the flux through the pathway is maintained. The changes to Eno1 and Pdc1 (in the presence of cadmium) are also considered to be part of the sulfur sparing response defined in response to oxidative stress (Fauchon *et al*., 2002). Eno1 is the sulfur-poor isoenzyme of enolase and Pdc1 is the sulfur-rich isoenzyme of pyruvate decarboxylase. Their differential regulation has been explained as a mechanism to conserve sulfur, a phenomenon called sulfur sparing (Fauchon *et al*., 2002), which is carried out by down-regulating the sulfur-rich isoenzyme and upregulating the sulfur poor isoenzyme.

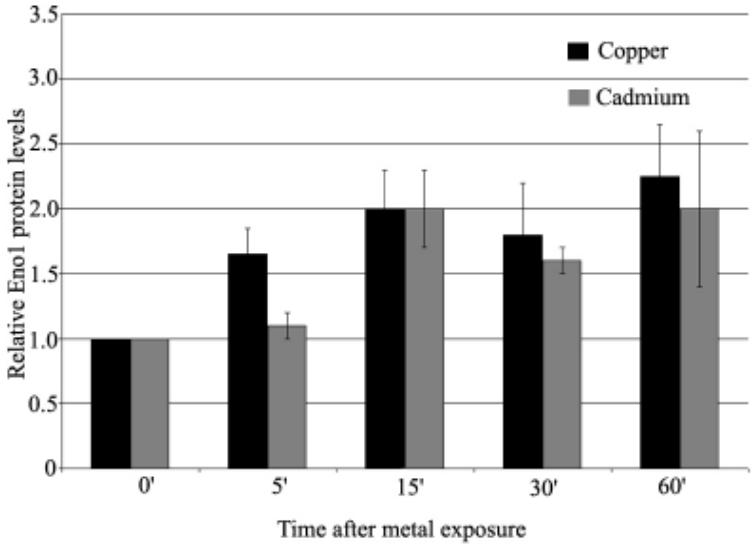

Figure 24. Eno1 levels increase in response to copper and cadmium exposure

Eno1 levels were analyzed by Cy-Dye DIGE before and after varying times of 10 mM copper or 30 mM cadmium exposure. Expression levels were normalized to control and expressed as relative units.

We also verified that the changes in the transcript levels of *pdc1 and eno1* reflected the changes in protein abundance, indicating a possible transcriptional basis for the observed differential expression (data not shown).

Cu-Zn superoxide dismutase is increased in response to copper, but not cadmium

Yeast cytosolic Cu-Zn superoxide dismutase, Sod1, is required to convert the more potent superoxides into the less potent hydrogen peroxide. The expression of Sod1 is mediated by various transcriptional activators including yAP-1, which is induced in response to Cd (Wemmie *et al*., 1994 and Wu *et al*., 1993), and Ace1, which is induced in response to Cu (Culotta *et al*., 1989 and Gralla *et al*., 1991). While Sod1 is established to be important for mediating resistance to copper by acting as an antioxidant and as a sink for copper (Culotta *et al*., 1995), its role in cadmium resistance is not defined. We found that Sod1 levels increased by 2 fold in response to copper, but not to cadmium (Figure 25). Even at higher concentrations, cadmium failed to induce Sod1 (Vido *et al*., 2001). This is consistent with findings that copper induces superoxides in the first hour, while cadmium, being a redox-inactive metal, in itself cannot (Fortuniak, 1996; Stohs et al., 2001) and does not (Nargund and Houghton, unpublished results). This confirms the long held thought that copper is akin to the superoxide generating menadione in its toxicity, while cadmium is more akin to H_2O_2.

Ubiquitin levels are increased by both copper and cadmium

Ubiquitin (Ubi4) is induced in response to stress and functions as a protein degradation tagging system. Conjugation of damaged proteins with ubiquitin marks it then for degradation by

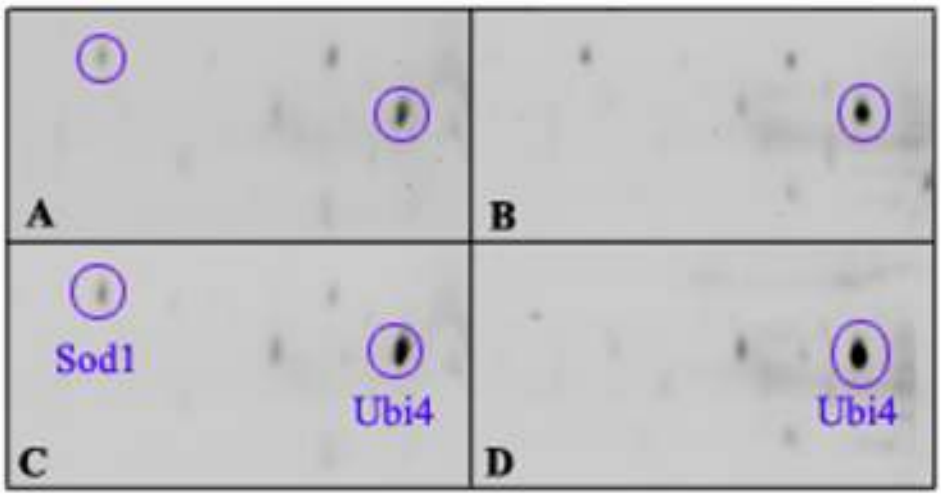

Figure 25. Sod1 increases in response to copper while Ubi4 increases in response to both copper and cadmium exposure

Proteins labeled with CyDyes before (A) and (C) and after an hour of 10 mM Cu (B) or 30 μM cadmium (D) exposure. Ubi4 (Ubiquitin) increases in response to copper and cadmium exposure as indicated by the series of spots circled in blue, whereas Sod1 (superoxide dismutase) increases only in response to copper.

ubiquitin mediated proteolysis. Having seen an increase in protein oxidation, which is synonymous with protein damage (Shacter, 2000), it was not entirely unexpected that we found ubiquitin levels to be increased in response to both copper and cadmium, indicating potentially increased protein damage and increased ubiquitin mediated proteolysis. It is noteworthy that the changes in ubiquitin are apparent in as little as 5 minutes after copper and cadmium exposure.

Amino-acid biosynthetic pathway proteins are repressed following cadmium exposure

Amino acid biosynthesis is repressed in response to cadmium exposure, as shown by a decrease in the levels of Hom2 (aspartic beta semi-aldehyde dehydrogenase), Ilv3 (dihydroxyacid dehydratase), Ilv5 (acetohydroxyacid reductoisomerase) and Aro8 (aromatic aminotransferase). Hom2 and Ilv5 have been shown to decrease in response to higher concentrations of cadmium (Vido *et al*., 2001) and Ilv5 and Ilv2 have been shown to decrease in response to H_2O_2, whose mode of toxicity is often compared with that of cadmium (Godon *et al*., 1998). Hom2 is involved in methionine and threonine biosynthesis, Ilv3 and Ilv 5 are involved in synthesis of the branched chain amino acids, leucine, isoleucine and valine while Aro8 mediates the synthesis of aromatic amino acids – phenylalanine, tryptophan and tyrosine. The decreases in amino acid biosynthesis, when considered together with an overall decrease in protein translation machinery, indicate a switch from a more energy expensive biosynthetic mode to a potentially energy conserving stress response mode.

Discussion

Reactive oxygen species (ROS) are inevitable by-products of aerobic respiration. Excess levels of ROS are generated upon metal exposure, which result in metal-induced oxidative stress. To offset the effects of metals and metal-induced oxidative stress, cells have been shown to orchestrate a cellular response by regulating gene and protein expression (Vido *et al*., 2001). In yeast, the proteomic response to the quintessential pro-oxidant, H_2O_2, is relatively well understood (Godon *et al*., 1998), while the proteomic response to the different metals remains relatively less well defined. Even so, it is apparent that some aspects of these stress responses are common to the different types of stressors, while other aspects appear to be quite specific. Consequently, in this series of studies on protein expression, we have attempted to characterize the proteomic responses of yeast to two different metals, copper and cadmium, which are known to differ in their mechanism of toxicity. In choosing to study the action of these two metals on protein expression, we also chose to investigate the effects of low and sub-lethal doses of each metal, primarily to ensure that any changes in pattern of protein expression that might be observed reflect a true profile of protein expression from living cells, and not a partial artifact resulting from the analysis of some indeterminable combination of living and dead cells within the culture. Moreover, mindful of the immediate and potentially short-lived oxidative responses of cells (Avery, 2000; chapters 1, 2 & 3), the expression profile of proteins was also determined at a series of time points within the first hour after metal exposure. In so doing, we have been able to confirm that, as with targeted protein oxidation, the cellular responses to each metal, at the level of protein expression are relatively rapid, reaching steady-state levels within a few minutes, while the persistence and degree of these changes vary with the magnitude of the stress.

We have also demonstrated that these changes in protein expression that result from exposure to sub-lethal doses of either copper and cadmium appear to occur in proteins that can be generally grouped as being involved in protein translation, carbon and sulfur metabolism, antioxidant enzymes and amino acid biosynthetic pathways.

There appear to be many areas of overlap, as well as differences, in the proteomic response to these two metals. Proteins involved in protein translation, including Eft1, Hyp2, Efb1 and Tif1 are decreased in response to both copper and cadmium. Protein translation is an energy expensive process and is, not surprisingly, among the first targets of both copper and cadmium-induced stress. A general decrease in multiple factors of the protein synthesis machinery indicates an overall decrease in the rate of protein synthesis, which has also been demonstrated in the response of yeast to H_2O_2 (Shenton and Grant, 2003; Shenton *et al*., 2006), metals (Jin *et al*., 2008) and various other oxidant stressors (Gasch *et al*., 2000 and Causton *et al*., 2001). More specifically, with respect to copper and cadmium, a decrease in the levels of the ribosomal proteins Rps5, Rps3 and RlaO is apparent after exposure to either metal. Such decreases not only reflect reduced ribosomal biogenesis, but also a decreased translational fidelity since these ribosomal proteins have also been shown to control translational fidelity during protein synthesis (Alksne *et al*., 1993 and Hendrick *et al*., 2001).

In addition to the overt depletion of proteins directly involved in protein synthesis and translational fidelity, synthesis of certain branched chain amino acids also appears to be affected by the presence of copper and cadmium, in that decreases in the levels of proteins Hom2, Ilv3, Ilv5 (which are all involved in the synthesis of various amino acids) are also observed. Depletion

of amino acids is known to increase the amount of uncharged tRNA, which (in turn) is known to activate a protein kinase, called Gcn2. An activated Gcn2 further inhibits translational initiation by phosphorylating eIF2α (Shenton *et al*., 2006). While we have not directly determined rates of protein synthesis, we can suggest that metal-induced stress inhibits protein synthesis, employing various strategies including decreasing protein translation factor levels, decreasing amino acid availability and thereby increasing levels of uncharged tRNA, and decreasing ribosome biogenesis. While it not possible at this stage to fully gauge the causes or consequences that such changes may ultimately have on cellular function, a decrease in protein synthesis is consistent with the apoptotic effects that H_2O_2 has been shown to initiate in yeast (Madeo, 2002; Madeo, 2004), which others in our laboratory have also recently shown to be elicited by low levels of cadmium and copper (Nargund *et al*, 2008 and unpublished observations).

In this regard, it is interesting to note that Ilv5, acetohydroxyacid reductoisomerase, an enzyme that is critical for the biosynthesis of isoleucine and valine is also involved in packaging and maintaining the mitochondrial genome into nucleoids (Bateman *et al*., 2002). A deletion of *ilv5* results in mitochondrial genome instability (Bateman *et al*., 2002), while an overexpression of Ilv5 is known to rescue mitochondrial instability *(*Zelenaya-Troitskaya *et al*., 1995*)*. Thus, it is tempting to speculate that a decrease in Ilv5 may also be responsible for, at least in part, depleting the cell's ability to maintain its mitochondrial integrity, this would have an adverse effect on mitochondrial genome stability, which is known to be a critical component in the aging process and part of the apoptotic program of cell death. While copper and cadmium elicit similar responses in the ribosomal and protein synthetic machinery, cadmium is unique in its induction profile of the sulfur metabolic enzymes, Cys3 and Cys4, both of which catalyze the synthesis of

cysteine, the amino acid precursor of the antioxidant tri-peptide, glutathione. Glutathione plays a critical role in the resistance to cadmium by sequestering cadmium in vacuoles that are then excreted by the cell as well as by forming thiol conjugates with proteins and, thereby, protecting them from damage (Li *et al*., 1997). In order to maintain or increase glutathione levels, yeast cells have been shown to orchestrate a number of responses. These include the transcriptional activation of proteins involved in glutathione synthesis (such as glutathione synthetase [Gsh1*)*), the yAP-1 mediated induction of glutathione reductase (Glr1) and also a phenomenon called the sulfur sparing response (Fauchon *et al*., 2002), in which crucial isoenzymes that are rich in sulfur (such as Pdc1 and Eno2) are down regulated in response to cadmium, while sulfur-poor isoenzymes (such as Pdc6 and Eno1) are simultaneously up regulated so as to channel the conserved sulfur towards producing more glutathione (Fauchon *et al*., 2002). In addition, we have further demonstrated a carbon metabolic response (Chapter 3) that is also thought to maintain reduced levels of glutathione under conditions of cadmium-induced oxidative stress. Both stress responses, apparently specific to cadmium, would be enhanced by an increase in Cys3 and Cys4, which code for cysteine beta synthetase and cystathione gamma lyase, respectively, and which catalyze the conversion of homocysteine to cysteine, the precursor to glutathione. In this study, we have demonstrated that both these enzymes are up regulated in response to higher concentrations of cadmium. Cys3 and Cys4 are transcriptionally activated by yAP-1 in response to H_2O_2 and Met4, following exposure to cadmium (Fauchon *et al*., 2002). Met4, along with yAP1, also partially controls expression of Gsh1, and is thus also involved in different aspects of glutathione production – sulfur assimilation as well as glutathione synthesis. Interestingly, we do not observe any change in the levels of Met6 and Met25, both of which have been shown to enhance sulfur assimilation. These proteins are only elevated in response to

higher cadmium concentrations (Fauchon *et al*., 2002) indicating that, while cysteine production can be rapidly elevated in response to low levels of cadmium (through increased expression of Cys3 and Cys4), any increase in sulphur assimilation requires higher levels of stress and/or prolonged exposure to cadmium.

Finally, the contrasting presence of ubiquitin and the copper-specific (Cu-Zn) superoxide dismutase provides a suitable distinction between shared and metal specific response of yeast to the two metals. Activation of ubiquitin is a common response to both copper and cadmium exposure. Ubiquitin conjugation to proteins "earmarks" them for proteolysis through the ubiquitin-dependent proteolysis pathway mediated by the 26S proteasome (Rivett, 1993; Coux *et al*., 1996 and Voges *et al*., 1999). This constitutes an effective means by which oxidatively damaged and unfolded proteins can be cleared away by the proteosome. Such an increase in ubiquitin synthesis, which is apparent in cells exposed to both copper and cadmium (Figure 25), essentially indicates an enhanced degree of protein unfolding and damage, entirely consistent with the protein damage that results from the increased levels of protein oxidation that we have previously shown to occur within an hour's exposure to metals (Chapters 1 and 3).

While elevation in the levels of ubiquitin is observed in response to copper and cadmium, the enhanced presence of Sod1 (Cu-Zn superoxide dismutase) is distinct to copper. Sod1 is thought to mediate Cu resistance by not only quenching superoxides but also by acting as a sink for excess Cu ions, thus specifically acting to buffer copper levels (Culotta *et al*., 1995). Cadmium, which elicits many cellular responses in yeast that overlap with the cellular responses to H_2O_2 does not initiate any increase in Sod1, even at higher concentrations (Vido *et al*., 2001).

Ace1 transcriptionally activates Sod1 during Cu response (Culotta *et al*., 1995). Interestingly, Sod1 is known to be a target gene of yAP-1 inducible by Cd. Even so, levels of Sod1 are not elevated during Cd response.

This series of preliminary proteomic analyses has identified some of the more immediate, first-line of proteomic changes that occur in yeast cells in response to sub-lethal levels of copper and cadmium. While the confines of the experimental structure limit the data output for analysis, we have shown that although copper and cadmium are entirely similar in their protein oxidation responses (Chapters 1 and 3), there are both apparent similarities and differences in their immediate responses at the level of the proteome. Both copper and cadmium are similar in their repression of protein synthesis machinery as well as their induction of ubiquitin, which indicates a series of shared responses to the presence of these two metals that are quite consistent with the newly obtained results in our laboratory, which suggest that even low levels of either cadmium or copper initiate a discernable apoptotic response that only becomes manifest a number of hours after any initial exposure to the metals (Nargund, 2008; Nargund and Houghton, unpublished observations). Such links, however, are associative at best, and still require further, more in depth, assessment and validation.

In contrast, each metal also appears to elicit metal-specific responses in the cell, which (where discernable) are consistent, to a large extent, with the differential transcriptional controls that have previously been identified for each metal (Culotta *et al*., 1989; Furst and Hamer, 1989 and Gralla *et al*., 1991). Such distinctions appear to be associated with the redox-potential of each metal, and are, perhaps, indicative of how yeast is able to clear the excess amounts of each

metal from the cell; the one (copper) through enhanced presence of Sod1, and the other (cadmium) through a more nuanced regulation of the sulfur metabolic enzyme, potentially to enhance glutathione synthesis. It is believed that the identification of such similarities and differences in the proteomic response of yeast to either cadmium or copper can only help to provide a backdrop for further characterization of oxidative protein and metabolic responses of yeast to the presence of low, sub-lethal concentrations of each metal, and broaden our understanding of the mechanisms of metal toxicity and cellular adaptive response to the presence of copper and cadmium.

References

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CHAPTER 5

GAPDH in Cadmium Toxicity in Yeast

Introduction

In the previous chapters we have shown that, in yeast, the glycolytic enzymes are specifically oxidized in response to metal induced oxidative stress. With protein oxidation capable of altering protein function (Shacter, 2000), we have further demonstrated that one of the discernable consequences of this targeted oxidation of glycolytic proteins is the re-routing of carbon metabolism away from glycolysis and into the pentose phosphate pathway (PPP), which serves as a cellular stress-resistance mechanism (Chapter 3). The centrality of the glycolytic process to the metabolism of *Saccharomyces cerevisiae* and, indeed, many other eukaryotic cells, would suggest that the shift in carbon flux toward the flow of carbon into the PPP need not be the only consequence of glycolytic enzyme oxidation, and that there are potentially a number of alternative consequences or additional repercussions to such targeted oxidation of glycolytic proteins in response to the presence of heavy metals. Indeed, given the importance of the glycolytic pathway to cellular function and the high abundance and ubiquitous presence of these proteins, it is no surprise that the highly ancient glycolytic proteins have provided a template for evolutionary change for some considerable time. A number of recent studies indicate that many glycolytic proteins can and do play a number of pleiotropic roles in a variety of cellular functions that are quite distinct from glucose metabolism (Kim, 2005). Hexokinase, enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are examples of such multifunctional proteins (Table 7) that have been shown to have a number of discrete activities in a series of

cellular processes. Such proteins use a number of different strategies (Figure 26) to switch between their various functions (Jeffrey, 2003), and are involved in aspects relevant to metal toxicity such as DNA repair, transcriptional response and apoptosis.

Of all these glycolytic proteins, GAPDH appears to be one of the more diverse enzymes, traditionally known to catalyze the NAD dependent conversion of glyceraldehyde-3- phosphate to 1, 3 biphosphoglycerate and NADH in glycolysis. It is a well characterized classic glycolytic enzyme encoded by a set of so-called "house-keeping genes", and is often used (quite erroneously, as it turns out) as an internal control in transcriptional expression studies. Aside from its well-defined cytoplasmic glycolytic function, GAPDH has been recently characterized as having a number of non-glycolytic functions, including telomere binding and stabilization (Sundararaj *et al*., 2004), histone transcription along with lactate dehydrogenase (Zheng *et al*., 2003), nuclear membrane fusion (Nakagawa *et al*., 2003), DNA repair (Baxi *et al*., 1995) and apoptosis (Chuang *et al*., 1996; Ishitani *et al*., 1997 ; Ishitani and Chuang 1996; Ishitani *et al*., 1996a; Ishitani *et al*., 1996b; Saunders *et al*., 1999). To switch between and among its various functions, GAPDH is known to either function as a monomer [DNA repair; (Mansur *et al*., 1993)], translocate to the nucleus [apoptosis, DNA repair, telomere binding; (Dastoor and Dreyer, 2001; Saunders *et al*., 1999; Hara *et al*. 2005)] and/or undergo post-translational modifications such as carbonylation [apoptosis; (Cahuana *et al*., 2004)], S-nitrosation [apoptosis; (Hara *et al*., 2005; Hara *et al*., 2006)] and variable formation of disulfide bond [nuclear translocation; (Cumming *et al*., 2005; Nakajima *et al*., 2007)]. GAPDH has also been observed to form multimeric complexes

Table 7. Non-Glycolytic functions of glycolytic proteins

Protein	Location	Function	Biological Significance	Reference
Hexokinase		Glucose sensor		
	Outer mitochondrial membrane	Apoptotic regulator	Binds to VDAC and prevents apoptotic death	Abu-Hamad *et al*., 2008
	Nucleus	Transcriptional regulation	In yeast, HXK2 represses genes for glucose metabolism in the presence of glucose	Rodriguez *et al*., 2001
			In plants, HXK1 controls the expression of photosynthetic genes in the presence of glucose	Chen, 2007
Enolase	Nucleus	Transcriptional regulation	An alternative splicing form of Eno1 represses MYC	Subramanian and Miller, 2000
	Mitochondria	tRNA transport	Co-factor in transporting tRNA into mitochondria	Entelis *et al*., 2006
GAPDH	Cell wall	Adhesion	Expressed in cell wall aids in cell-cell and cell-matrix adhesion	Barbosa *et al*., 2006
	Mitochondria	Apoptosis	Regulates mitochondrial membrane permeabilization during apoptosis	Tarze *et al*., 2007
	Nucleus	Transcriptional regulation	Histone gene expression – part of a coactivator complex	Zheng *et al*., 2003
	Nucleus	Nuclear membrane assembly	Participates in nuclear membrane fusion	Nakagawa *et al*., 2003
		Telomere maintenance	Maintains telomere structure	Sundararaj *et al*., 2004
		DNA repair	Recognizes and removes fraudulent nucleotides	Krynetski *et al*., 2003
		Apoptosis	Protein levels increase, especially in nucleus	Ishitani *et al*., 1996a; Ishitani *et al*., 1996b, Sawa *et al*., 1997 and Hara *et al*., 2005

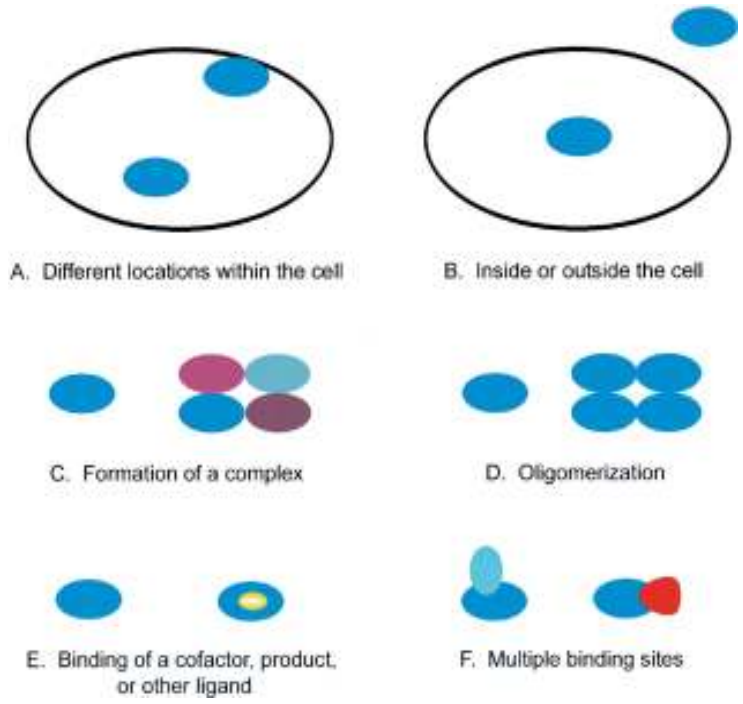

Figure 26. Strategies used by proteins to switch between different functions

Pleiotropic proteins use various strategies to switch between various functions. GAPDH is known to follow at least 2 of these strategies – adopting different subcellular locations (A) and formation of a complex (C) (Jeffery, 2003)

with other proteins in order to participate in gene transcription. Thus, GAPDH is a part of a heteromeric co-activator complex required for histone 2A transcription along with Oct-1 and lactate dehydrogenase (Zheng *et al*., 2003). In all such studies performed on GAPDH in higher eukaryotes, the enzyme was found to be upregulated in apoptotic cells (Ishitani, 1997; Sunaga, 1995). Moreover, any block in this increased expression using siRNA actually prevented apoptosis (Ishitani et al., 1996a; Ishitani et al., 1996b), which suggests that increases in GAPDH levels are not only associated with apoptosis, but are necessary for apoptosis to occur. Subsequently, GAPDH carbonylation and S-nitrosation have also been seen to precede apoptosis (Cahuana, 2004) in these cells. Although the exact role that GAPDH plays in the apoptotic process is still not fully understood, from the outset it was apparent that, in order for GAPDH to participate in apoptosis, its translocation into the nucleus would be prerequisite to the process (Ishitani, 1996a; Ishitani, 1998). The nucleotide sequence of GAPDH, however, lacks a nuclear localization signal (NLS) and there is increasing evidence that GAPDH is unable to translocate to the nucleus by itself, fueling speculation that it uses a partner protein, SiaH and its NLS, to gain access into the nucleus at least in neuronal apoptotic events that are subject to S-nitrosative stress (Figure 27; Hara *et al*., 2005). Intriguingly, GAPDH is by itself unable to associate with its nuclear translocating partner, SiaH. It does so only upon undergoing stress-induced post-translational modification such as S-nitrosation. Thus, studies in higher eukaryotes suggest that, at least in neuronal cells, GAPDH is a potential sensor of nitrosative stress and oxidative stress and, thereafter, a mediator of apoptosis (Hara *et al*., 2005; Cahuana *et al*., 2004). Since apoptotic responses and mechanisms are not completely conserved between yeast and higher eukaryotes, whether GAPDH plays any role as an oxidative stress sensor and mediator of apoptosis in yeast remains to be seen.

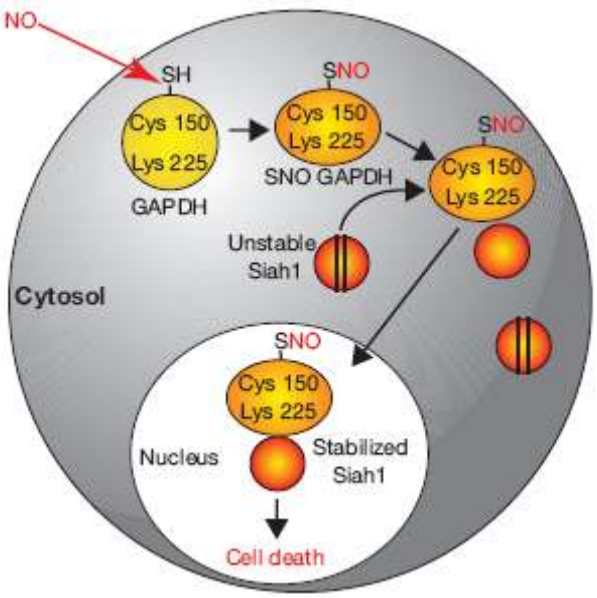

Figure 27. Schematic cell death cascade showing NO-S-nitrosation-GAPDH and SiaH

Nitric oxide causes the S-nitrosation of GAPDH at Cys 150, which potentiates binding to the unstable SiaH at Lys 225. Binding to GAPDH stabilizes SiaH and helps the two proteins translocate to the nucleus. Once in the nucleus, SiaH triggers cell death by degrading nuclear proteins (Hara *et al*., 2005).

It is pertinent then, to study the potential role of GAPDH in this process, because *Saccharomyces cerevisiae*, being a model system in the study for many stress related conditions, is also one of the few unicellular organisms that is capable of undergoing apoptosis (Madeo, 1997; Madeo, 2004). Even so, the current level of understanding of apoptosis in yeast needs more clarity. Recently, others in the laboratory have demonstrated that wild-type yeast cells undergo apoptosis in response to cadmium (Nargund *et al*., 2008). Furthermore this response to cadmium was abolished in yeast cells deleted for *tdh3*, a gene that codes for the major isoenzyme of GAPDH (*tdh3*Δ) (McAllister and Holland, 1985). Thus, although the major isoenzyme of GAPDH, Tdh3 is known to be required for apoptosis in yeast and is one of the major isoenzymes that is carbonylated in response to cadmium (as well as copper and chromium; Shanmuganathan, 2004; Sumner, 2005), it is unclear as to how this isoenzyme actually participates in the apoptotic process. In this chapter, therefore, we have attempted to characterize the cadmium-induced changes in GAPDH *viz.*, abundance, protein oxidation, protein fragmentation, oligomeric status and nuclear translocation, in an effort to understand more thoroughly the role of GAPDH in cadmium-induced apoptosis. To do so, we have opted to study GAPDH in a more extended time frame (up to 3 hours after metal exposure) since apoptosis is manifested only after such an extended period of metal exposure. In our analyses, we find that GAPDH levels increase in the cell in response to protracted exposure of low levels of cadmium, and that there is also an increased localization of GAPDH in the nucleus. Intriguingly, these changes occur in an isoform specific manner. Furthermore, in trying to ascertain specific changes in GAPDH configuration within the cell in response to cadmium exposure, we found that the oligomeric interactions that are normally maintained by GAPDH are abrogated following the addition of cadmium. These findings enhance our understanding of the role that GAPDH plays in the response of cells to low

levels of cadmium and cadmium-induced apoptosis, as well as shedding light on the potential use of the different isoforms of GAPDH to participate in multiple cellular functions.

Materials and Methods

Strains, media and metal exposure

Yeast cells BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were routinely maintained on YEPD agar. Experimental cultures were inoculated from 24-hr starter cultures derived from a single colony and were grown overnight in YEPD broth to an OD of ~2.0 at 30°C with orbital shaking 120 rev min^{-1}. Cadmium was added to the culture at this stage to a final concentration of 30 μM and allowed to grow for up to 3 hours.

Total protein extract preparation

Cells were harvested by centrifugation (2000 x g, 5 minutes, 4°C) before and during metal exposure, and washed twice in cold, sterile distilled water. The washed cell pellets were resuspended in 200 μl of lysis buffer (100 mM Tris.Cl pH 7.4, 20 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 0.264 mg ml^{-1} aprotinin, 1 mM PMSF, 10% (v/v) glycerol). The cells were then disrupted with glass beads of 0.5 mm diameter in a mini-bead beater 8 (Biospec products). The cell debris was removed by centrifuging the cell and bead mixture (20, 000 x g, 10 minutes, 4°C). The supernatant was collected; the protein concentration was determined [using the 2D quant kit (GE Life Sciences)] and the sample was stored at -20°C before further analysis.

Cytoplasmic and nuclear extract preparation

Cells were harvested by centrifugation (2000 x g, 5 minutes, 4°C) before and during metal exposure. Cell pellets were weighed and washed twice in cold, sterile distilled water before being resuspended in pretreatment buffer (1 ml / g wet cell weight) (50 mM Tris, pH 7.5, 30 mM DTT) and incubated at 30°C for 15 minutes. The pretreated cells were harvested (2000 x g, 10 minutes, 4°C) and resuspended in spheroplast buffer (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol) (1 ml / g wet cell weight) containing Zymolyase 20T (2.5 mg / g wet cell weight) and incubated at 30°C for 1.5 hrs with intermittent gentle shaking every 15 minutes. The spheroplasts were then gently washed thrice in spheroplast buffer (1 ml / g wet cell weight). After each wash, the spheroplasts were harvested by centrifugation (1500 x g, 12 minutes, 4°C). After the final wash, the spheroplasts were resuspended gently in lysis buffer (1ml / g wet cell weight) (20 mM HEPES/KOH (pH 7.4), 0.6 M sorbitol, 0.5 mM PMSF and 1μg/μl each of pepstatin, aprotinin, leupeptin) and allowed to sit on ice for 30 minutes. At the end of the incubation, the spheroplasts were lysed (50 strokes) in a Potter-Elvehjem glass-teflon homogenizer. 20% (w/v) triton-X 100 (1 ml per 15 ml lysate) was added to the homogenate and mixed by inversion many times. The lysate was centrifuged (1000 x g for 10 minutes, 4°C) and the resulting pellet (triton-X insoluble fraction) and the supernatant (triton-X soluble fraction) were stored at -20°C. Nuclear proteins were then extracted from the pellet (triton-X insoluble fraction) by agitating at 800 rpm for 15 minutes at 4°C with nuclear lysis buffer (20 mM HEPES, pH 7.9, 0.4 N NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1μg/μl each of pepstatin, aprotinin and leupeptin). The lysate was then centrifuged (13,800 x g for 20 minutes at 4ºC) to obtain the nuclear extract. The triton-X soluble fraction was centrifuged at 100,000 x g for 1 hour in the ultracentrifuge to

obtain the cytosolic fraction. Protein concentrations were determined using the 2-D quant kit (GE Life Sciences) and stored at -20ºC before further analysis.

SDS-PAGE and Western blotting

Yeast lysates were subjected to 12% SDS-PAGE and transferred to Hybond nitrocellulose membranes. For Western blotting, nitrocellulose membranes containing the proteins were blocked with PBS-0.1% Tween-20 containing 5% skim milk for 1 hour at RT. Rabbit anti-Adh (Chemicon) was used to identify alcohol dehydrogenase at 1: 5000 dilution in blocking buffer; mouse anti-nsp1 (EnCor) was used to identify nuclear surface porin at 1: 3000 dilution; rabbit anti-GAPDH (Sigma) was used to identify glyceraldehyde phosphate dehydrogenase at 1:10,000 dilution and rabbit anti-DNP antibody was used at 1:2500 dilution. Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma); goat anti-mouse IgG conjugated to HRP (Sigma) or donkey anti-chicken IgG conjugated to HRP (Chemicon) were used at 1:5000 dilution and the bands were detected by enhanced chemiluminescence (Amersham).

RT-PCR

Yeast cells were exposed to 30 μM Cd and harvested before and after 1, 2 and 3 hours of Cd exposure. RNA was extracted from these cells using the RNEasy mini kit (Qiagen). Extracted RNA (8-10 ug) was treated with RNAse free DNAse I (1U / μg RNA) in DNase digestion buffer (10 mM Tris, pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) for 30 minutes at 37ºC. Subsequently, DNaseI was inactivated by incubating the samples at 75ºC for 10 minutes. 1.5 μg of DNase treated RNA was used for RT-PCR using the Ready-To-Go RT-PCR kit (GE). *tdh3* specific

primers were used (Primer1: 5' – AACGACCCATTCATCACCAACGAC-3'; Primer 2: 5'-TGGAAGATGGA-GCAGTGATAACAA-3') as designed by Kang *et al*. (2000). The template was amplified for 24 cycles and the RT-PCR products were analyzed on a 1.5% agarose gel.

Diagonal-Gel Electrophoresis

Protein fractions to be analyzed were first separated on a 0.75 mm thick, 12.5% homogenous SDS-PAGE gel under non-reducing conditions. After electrophoresis, the protein bands were stained in Coomassie Brilliant Blue and destained with destaining solution (40% methanol and 10% acetic acid). The lanes were then excised and equilibrated for 10 minutes in SDS-equilibration solution containing 1 M DTT and bromophenol blue. After equilibration, the lane was then horizontally inserted between the glass plates of a 1.5 mm thick 12.5% polyacrylamide gel and separated. The gels were then either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membranes and probed with anti-GAPDH antibody as defined earlier.

Results

General

Previous findings in our lab (Nargund *et al*., 2008) have shown that cadmium at a concentration of 30 μM is non-lethal to yeast cells in the first hour of exposure. However within this time, cadmium was able to induce apoptosis in a small sub-population of cells, which was only manifested after two to three hours after metal exposure. Accordingly, while the studies presented in this dissertation thus far have exclusively involved the "immediate" metabolic and cellular responses of yeast to low concentrations of heavy metals (within the first hour), any

subsequent investigation of proteins that may be involved in an apoptotic response must involve a wider timeline, Consequently, we chose to analyze changes in GAPDH in yeast cells before and after 3 hours of cadmium treatment keeping in mind that changes in GAPDH may not be apparent in a smaller time frame (1 hour) such as that employed earlier.

mRNA levels of GAPDH (tdh3) increase following cadmium exposure

Higher eukaryotic cells such as neuronal cells and non-neuronal mammalian cells that require GAPDH for apoptosis are seen to exhibit upregulated levels of GAPDH (Ishitani *et al*., 1996a; Ishitani *et al*., 1996b; Ishitani *et al*., 1997 and Chen *et al*., 1999). Indeed the increase in GAPDH is almost always seen in systems that require GAPDH for apoptosis. Having seen a requirement of GAPDH for cadmium induced apoptosis in yeast, we assessed whether the transcript levels of GAPDH changed within the cell in response to metal treatment by RT-PCR. The RNA extracted from yeast cells before and after Cd exposure was treated with RNAse free DNase1 to remove all DNA contamination. The RNA concentration was remeasured and RT-PCR was carried out on these DNAse treated RNA templates and the products were analyzed by agarose gel electrophoresis When primers specific to GAPDH that not discern between the various isogenes were used, transcript levels of tdh increased modestly by 15% within one hour of cadmium treatment before decreasing to levels seen before cadmium treatment (Figure 28A and B). To finesse this analysis, primers were chosen for RT-PCR of *tdh3* so as to exclude *tdh1* and *tdh2*, the genes encoding the other 2 isoenzymes of GAPDH (Kang *et al*., 2000) and the RT-PCR analysis was repeated (Figure 28C). The transcript levels were quantified using Fuji Multigauge software V. 2.0 and the changes are depicted in Figure 28D. It was found that *tdh3* transcript levels increased by about 1.6 fold after 1 hour of Cd exposure before stabilizing to

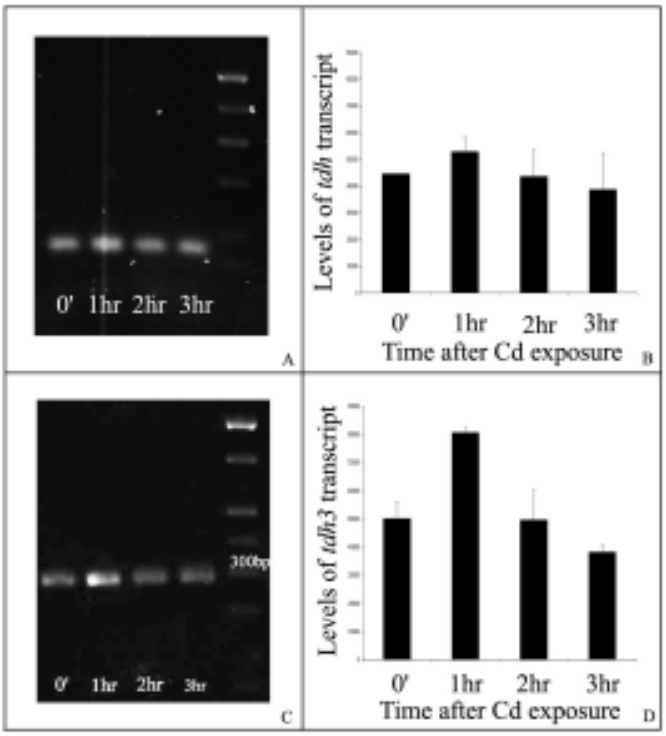

Figure 28. Levels of GAPDH transcript as seen after 30 μM cadmium exposure

GAPDH RNA was amplified by RT-PCR with 24 cycles of amplification using gene specific isogene indistriminate primers for *tdh* (A) and using gene specific, *tdh3* isogene primers (C). Equal volumes of RT-PCR product were analyzed on a 1.5% agarose gel. Transcript levels in A and D respectively were quantified using Fuji MultiGauge and depicted (B and D).

levels seen before cadmium exposure. Thus, when isogene specific primers were used, the increase in transcript levels was greater than when indiscriminate primers were used indicating that among the isogenes coding for GAPDH, it is *tdh3* which is subject to a greater change upon cadmium treatment.

GAPDH is upregulated in the total protein lysates after cadmium exposure in an isoform specific fashion

Having seen an increase in levels of *tdh3* mRNA levels, we sought to verify whether this transcriptional increase was reflected at the level of protein. To do so, we determined GAPDH levels by immunoblotting in total protein extracted from cells (before and after 30 μM cadmium exposure). As can be seen in Figure 29A, the levels of GAPDH progressively increased after 1, 2 and 3 hours of Cd exposure. The band intensities were quantified using Fuji Multigauge software to reveal that GAPDH levels increased by about 2 fold following 2 and 3 hours of Cd exposure (Figure 29B). To ascertain that the changes in GAPDH are not an artifact introduced as a result of varied loading, the same blots were also probed with anti-adh antibody for alcohol dehydrogenase as a loading control. Adh levels remained unchanged in the different samples (Figure 29C).

Having thus seen an increase in GAPDH protein levels in the whole-cell lysate, we sought to determine if this increase was isoform specific. We therefore subjected total protein fractions to 2D electrophoresis in the pI range of 3-10, followed by separation on 12.5% SDS-PAGE. GAPDH detected by immunoblotting showed that the isoformic distribution of GAPDH changed after Cd treatment (Figure 29D). The isoelectric point of GAPDH increased after Cd exposure

and the pI profile shifted more to the basic end. Also, while all the isoforms increased after cadmium exposure, the relative increases were greater in the acidic isoforms with a slightly lower molecular weight. The identity of these new spots (indicated by arrows) was confirmed by mass spectrometry to be GAPDH. However, due to the high sequence similarity between the isoenzymes of GAPDH, Tdh3 and Tdh2, it was not possible to distinguish between these two forms by either Western blotting or mass spectrometry. Even so, based on previous studies that have shown Tdh2 to migrate closer to, but below, Tdh3 (Mitsuzawa *et al*., 2005), it is possible that these lower molecular weight forms of GAPDH that are specifically upregulated after Cd exposure also include forms of Tdh2.

GAPDH is increasingly carbonylated following cadmium exposure

We and others have shown that cadmium exerts its toxic effects through oxidative stress mechanisms (Stohs and Bagchi, 2000; Chapter 3). Oxidative stress causes post-translational modifications in proteins and in this regard GAPDH has been shown to undergo carbonylation (Figure 15, chapter 3), S-thiolation (Shenton and Grant, 2003) and ADP-ribosylation. Among these modifications, carbonylation is a prominent and permanent protein change, and is easily detectable by coupling the protein with DNPH and is apoptotically relevant (Cahuana *et al*., 2004). We therefore analyzed carbonylation in GAPDH following cadmium treatment by derivatizing the proteins with DNPH. Carbonylated proteins were then detected by immunoblotting with antibodies against DNPH. We observed that before cadmium exposure,

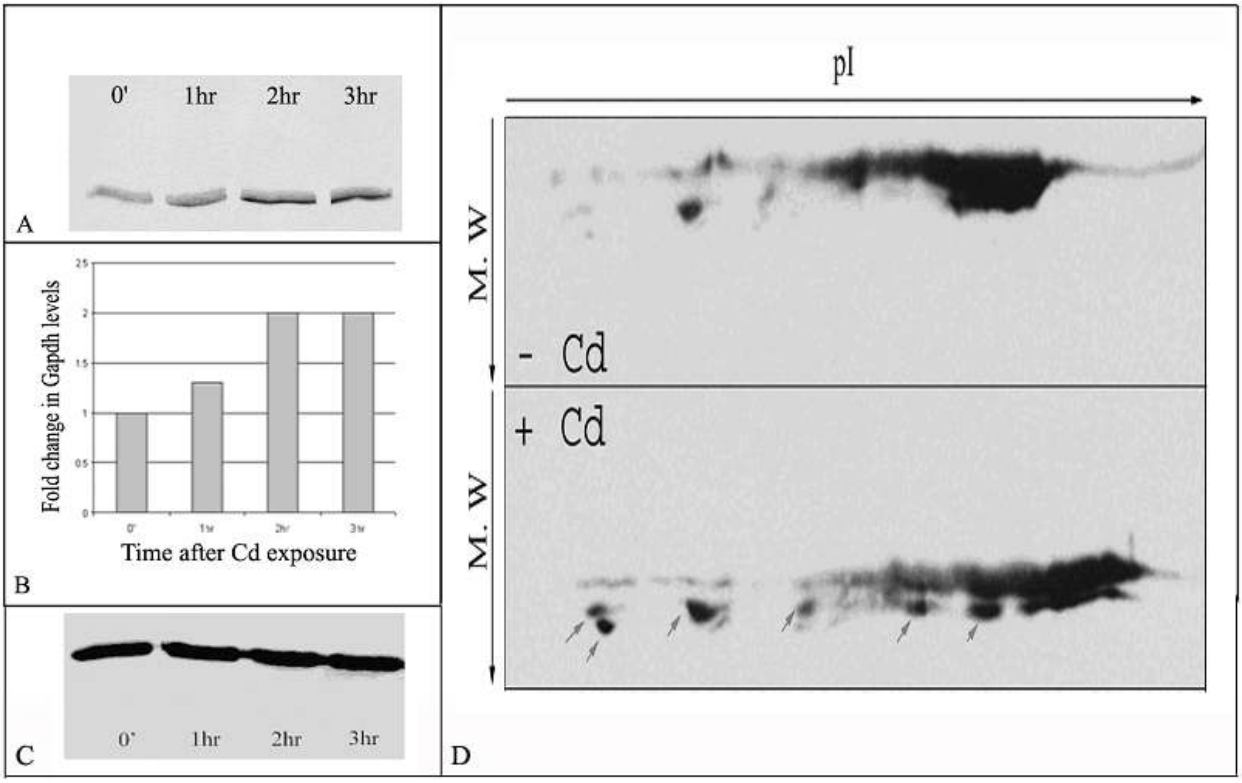

Figure 29. Levels of GAPDH in yeast cells increase after 30 μM cadmium exposure in the whole cell while Adh remains unchanged

Proteins were extracted from yeast cells before (0') and after varying times of cadmium exposure. GAPDH in 30 μg of total protein was detected by Western blotting using anti-GAPDH as primary antibody (1: 5,000) and anti-chicken-HRP conjugated secondary antibody (1: 5,000). The bands were detected by chemiluninescence (A). The band pixel intensities were analyzed using Fuji MultiGauge software, normalized to the levels of GAPDH at 0' and depicted (B). As an internal standard, these extracts were assessed for alcohol dehydrogenase using anti-adh antibodies (Chemicon, 1: 5,0000) (C). Total protein obtained from cells before and after 3 hours of cadmium exposure was separated by 2D-electrophoresis. The 2D gels were then probed with anti-GAPDH antibody (1: 5000) and secondary antibody, conjugated to HRP (1: 5000). The blots were then visualized using chemiluminescence. The forms of GAPDH upregulated after Cd exposure are indicated by arrows (D).

very little GAPDH was carbonylated while after cadmium exposure, carbonylation levels in GAPDH increased (Figure 30). Interestingly, only Tdh3 could be clearly identified as being oxidized, while none of the various isoforms of Tdh2 appeared to bind the DNPH antibody.

Cytosolic and nuclear GAPDH increase after cadmium exposure

To participate in apoptosis, GAPDH is known to translocate to the nucleus (Hara *et al*., 2005, Saunders *et al*., 1999; Ishitani *et al*., 1996a; Figure 27). In some instances, this translocation is even shown to be isoform specific (Saunders *et al*., 1999 and Sunderaraj *et al*., 2004). To assess whether this happens in yeast, sub-cellular protein fractions of yeast were prepared. Yeast cells were converted into spheroplasts and then homogenized to separate the nuclei away from the cytoplasm as detailed in Materials and Methods. Prior to lysis, the nuclei were stained with DAPI (a DNA binding dye) and observed under the fluorescence microscope. DAPI staining of the prepared nuclear fractions was positive (Figure 31), demonstrating that the nuclei were indeed concentrated in this fraction. Subsequently, the cytosolic and nuclear protein fractions were probed by immunoblotting with cytosolic anti-adh (alcohol dehydrogenase) and nuclear anti-Nsp1 (anti- nuclear surface porin) antibody markers to determine the integrity of the fraction. The cytosolic fraction was positive for Adh and negative for Nsp1, confirming cytosolic integrity (Figure 32A and B). However, the nuclei although positive for Nsp1 also exhibited some residual cytosolic contamination (< 20%) as revealed by the presence of Adh (Figure 32A and 32B).

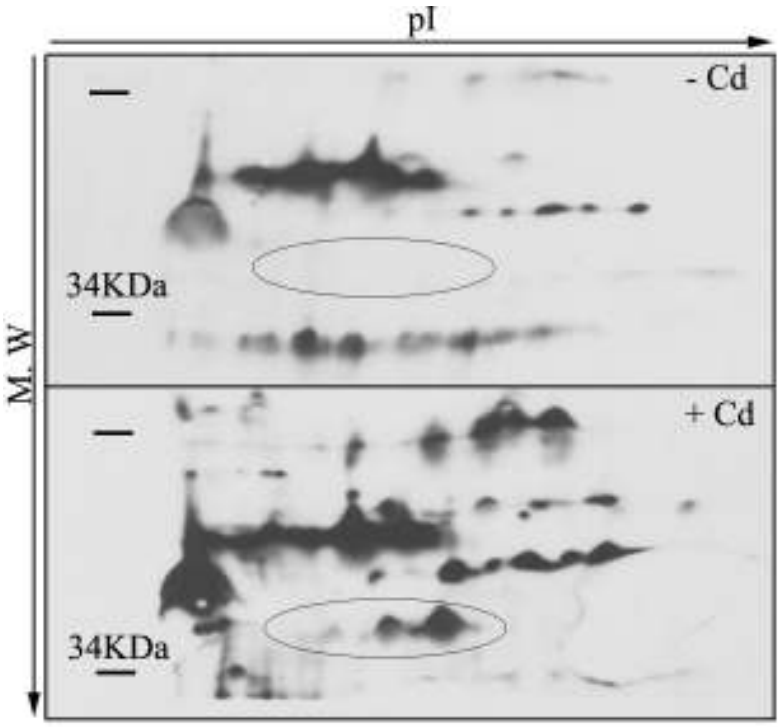

Figure 30. GAPDH is increasingly oxidized (carbonylated) in the whole cell after cadmium exposure

Proteins were extracted from yeast cells before (0') and after 3 hrs of cadmium exposure and separated by 2D electrophoresis on pI 3-10 IPG strips and derivatized with 2-DNPH to detect carbonylated (oxidized) proteins. Oxidized proteins were detected by Western blotting using anti-DNPH as primary antibody (1: 2,500) and anti-rabbit-HRP conjugated secondary antibody (1: 2,500). The spots were detected by chemiluminescence. The position of GAPDH isoforms are indicated by ellipses.

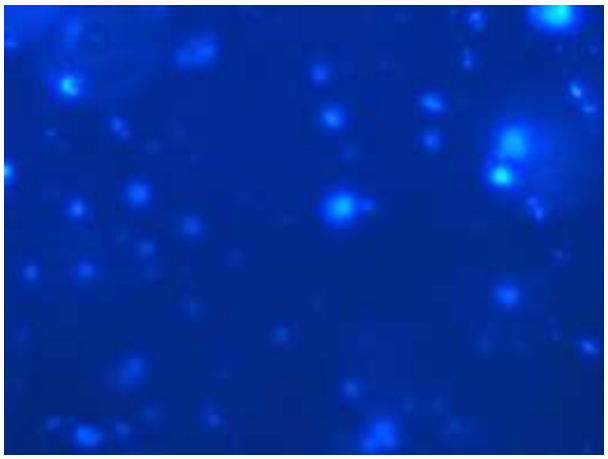

Figure 31. DAPI staining of nuclei

The isolated nuclei were stained with DAPI and mounted in antifade and observed under fluorescent microscope.

Having thus qualitatively determined the quality and integrity of the cytoplasmic and nuclear extracts, we assessed these sub-cellular fractions for the presence of GAPDH by immunoblotting. As shown in Figure 32C, the levels of GAPDH increased following cadmium exposure by 2 fold in the cytosol. In the nuclear fraction, the levels of GAPDH were lower in the cadmium untreated control compared to the cytosol but increased by more than 6 fold after Cd exposure, surpassing the fold change in the cytosol. These Cd treated nuclei displayed only residual (< 20%) cytosolic carry over compared to purified cytosol and yet showed 3 fold more GAPDH than Cd treated cytosol. This exaggerated fold change in GAPDH, present in the Cd treated nuclei while the endogenous control Adh remains unchanged, is strongly indicative of nuclear translocation of GAPDH.

The nuclear forms of GAPDH were earlier shown to be distinct, with a different isoelectric point compared to the cytosolic GAPDH (Saunders *et al*., 1999 and Sunderaraj *et al*., 2004). To determine whether this was true in yeast, 2D electrophoresis was undertaken on these cytosolic and nuclear extracts followed by Western blotting for GAPDH (Figure 32D and 32E). The isoformic profile of the cytosolic extract showed a very similar profile as that of total GAPDH. The 2D profiles were able to verify that there was a shift in the pI of GAPDH and a 2-fold overall increase in levels. In the nuclear GAPDH, only the most abundant GAPDH forms were present to begin with, and after cadmium exposure there was an increased presence of the smaller molecular weight GAPDH (as indicated by arrows). However, no form of GAPDH was uniquely present in either the nucleus or cytoplasm.

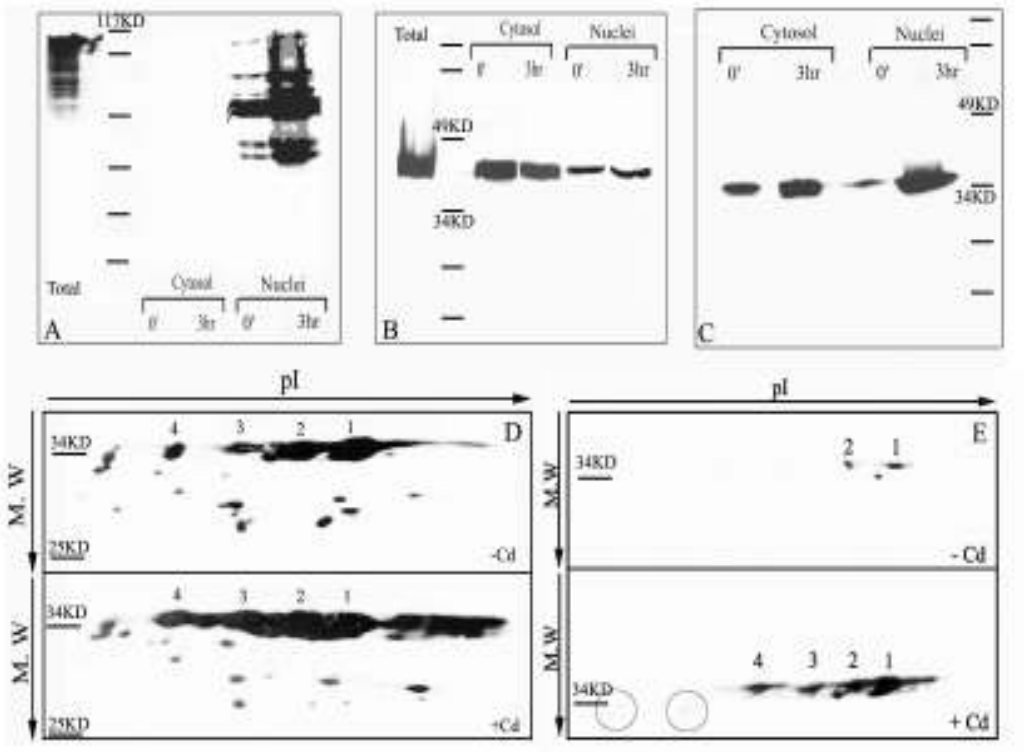

Figure 32. Cytosolic and nuclear GAPDH increase after Cd treatment
Subcellular protein fractions were prepared as outlined in Materials and Methods and assessed for the presence of nuclear marker, nuclear surface porin, Nsp1 using anti-nsp1 antibody. The 100 KDa band corresponding to Nsp1 was present in the total lysate and the nuclei and absent in the cytosol (A). The same fractions were probed with the cytosolic marker, alcohol dehydrogenase using anti-adh1. The 42 KDa band corresponding to Adh1 was seen in the total lysate and the cytosol. The nuclear fractions also showed Adh presence albeit to a lower extent (<20%) (B). The cytosol and nuclei were assessed for the presence of GAPDH using anti-GAPDH antibodies. The protein levels of which increased by 2 fold in the cytosol and by >5 fold in the nuclei after Cd exposure (C). The cytosolic fractions (D) and the nuclear fractions (E) were separated by 2D electrophoresis and then Western blotting with anti-GAPDH antibodies were undertaken to determine the isoformic profile of GAPDH before (top panels) and after Cd exposure (bottom panel). The major isoforms are numbered from the more basic (1) to the more acidic (4). Lower molecular weight GAPDH in the nuclei are indicated by circles.

GAPDH undergoes fragmentation in response to cadmium exposure

While ascertaining the levels of GAPDH by Western blotting in protein lysates, we noticed smaller protein bands between 20 KDa and 35KDa were reactive with anti-GAPDH antibody, as indicated in Figure 33. Curiously, these fragments were absent in the cytosolic fraction and present only in the nuclear fraction (Figure 33C). During apoptosis it is known that a series of proteins are cleaved by specific, apoptotic proteases called caspases (Cohen, 1997; Thornberry and Lazebnik, 1998). Recently, in macrophages undergoing apoptosis, GAPDH was shown to be a target of caspase-1 (Shao *et al*., 2007). In yeast, the gene *yca1* encodes the only caspase (Madeo, 1999). To ascertain whether these bands were products of Yca1 mediated proteolysis, GAPDH was identified by Western blotting protein lysates from yeast cells deleted for *yca1*. There was no difference in the band profile of wild-type and *yca1*Δ yeast, indicating that these products are not caspase dependent (Figure 33D). It remains to be seen whether these fragments are non-caspase mediated proteolytic products or mere oxidative stress mediated protein break-down products since it is known that protein oxidation itself is capable of cleaving peptide backbones in proteins (Shacter, 2000). Nevertheless, the fact that these fragments are present in the nuclei and absent in the cytosol (Figure 33C) is in itself quite intriguing, and adds an additional variant that is potentially relevant to metal toxicity and, perhaps, apoptosis.

Oligomeric interactions of GAPDH are abolished after cadmium exposure

Protein oxidation is known to change protein tertiary structure (Shacter, 2000). As a consequence, it is conceivable that protein-protein interactions would be compromised. To investigate this hypothesis, we analyzed GAPDH presence in protein lysates obtained from cells before and after cadmium exposure under denaturing (+ SDS), non-reducing (- DTT) conditions.

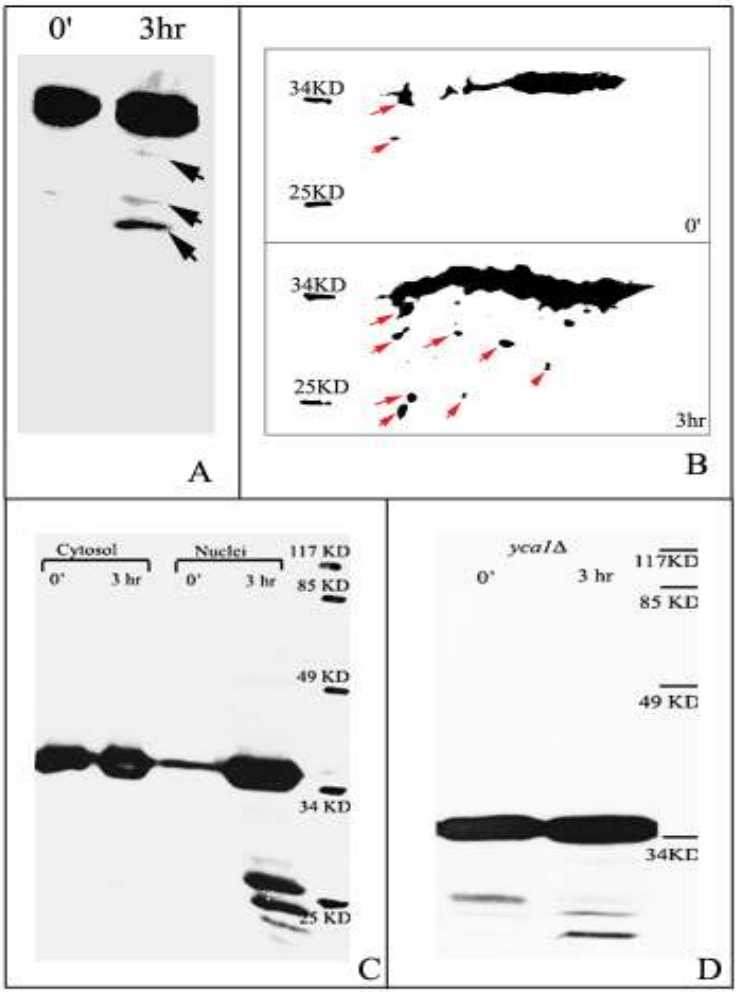

Figure 33. GAPDH is increasingly fragmented after Cd exposure

GAPDH was detected by Western blotting in protein extracts prepared from yeast cells before and after 3hrs of 30□M Cd exposure. Smaller molecular weight-GAPDH are denoted by arrows in 1D Western blots (A) and 2D Western blots (B). The sub-cellular fractions cytosol and nuclei obtained from cells before and after metal exposure were similarly probed to identify GAPDH and it's fragments (C). GAPDH and it's fragments as seen in yca1□ yeast cells before and after Cd exposure (D)

In cadmium untreated cells, a number of higher molecular weight bands reactive with anti-GAPDH antibody were detectable in non-reducing conditions (Figure 34, left lane) and not under reducing conditions (Figure 28A). This shows that under normal conditions, GAPDH interacts with itself and/or other proteins through disulfide linkages. Under cadmium treated conditions, no band but the monomer is detectable under both reducing and non-reducing conditions, indicating that those interactions normally maintained by GAPDH are abrogated after cadmium exposure.

To verify that these oligomeric anti-GAPDH reactive bands were indeed GAPDH and not any non-specific artifact, diagonal redox two-dimensional electrophoresis was performed as described in Materials and Methods. In this method, higher molecular weight complexes dissociate into their components and run according to their molecular weight below the diagonal. Immunoblotting of diagonal electrophoresis runs from lysates of cadmium untreated cells (control) revealed a majority of GAPDH to be present on the diagonal. Also present were more GAPDH reactive, 35 KD spots situated below the diagonal that were a result of GAPDH oligomers dissociating under the reducing conditions in the second dimension (shown by black arrows). The smaller fragments of GAPDH specifically induced upon Cd treatment were also seen along the diagonal below the main 35KD GAPDH spot. On the other hand, the lysates from cadmium treated cells only contained the monomeric GAPDH on the diagonal (Figure 34, right panel). This confirms that the disulfide mediated interactions maintained by GAPDH with itself and / or other proteins are abolished after cadmium exposure.

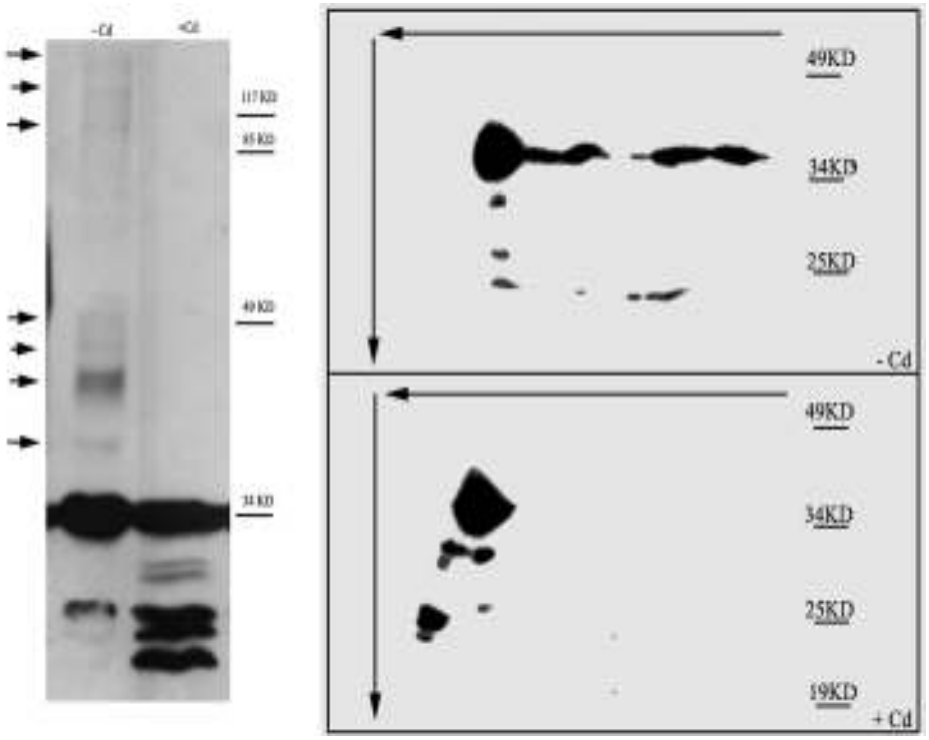

Figure 34. Cd affects oligomerization of GAPDH

Left: GAPDH was identified by Western blotting of total protein lysates obtained from yeast cells before and after Cd treatment under denaturing (+SDS) and non-reducing (- DTT) conditions. Right: GAPDH was identified by Western blotting of total protein lysates obtained from yeast cells before and after Cd treatment after diagonal-gel electrophoresis. The first dimension was carried out in the absence of DTT (horizontal) and the second dimension was carried out in the presence of DTT. The presence of higher molecular weight GAPDH in the top panel are shown as a series of spots on the horizontal below the diagonal.

Discussion

Glycolytic proteins as a group are an ancient and highly conserved family of proteins that are intriguingly an important target of oxidative stress (Collussi *et al*., 2000; England *et al*., 2004; Shenton and Grant, 2003; Shanmuganathan *et al*., 2004 and Sumner *et al*., 2005). Glycolytic enzymes were among the first proteins that evolved in an originally anaerobic environment. Therefore, it is not too surprising that when the environment switched to aerobicity and oxygen came to being present, these same glycolytic proteins and the process of glycolysis have been employed as oxidative stress signals (England *et al*., 2004; Blackburn *et al*., 1999 and Collussi *et al* 2000). Aside from their traditional role in glycolysis, these glycolytic proteins have evolved multiple functions, often in non-cytoplasmic locations (Table 7). Their ubiquitous nature and high abundance, coupled with the fact that they are one of the most ancient proteins, has given ample time in evolution for the cells to tinker with what is already available and provide additional functions to these same proteins (Jeffrey, 1999 and Sriram *et al*., 2005). One such glycolytic protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), has been newly characterized with diverse activities such as DNA repair (Baxi *et al*., 1995), telomere maintanence (Sundararaj *et al*., 2004), membrane fusion (Nakagawa *et al*., 2003), transcriptional regulation (Zheng *et al*., 2003) and apoptosis (Chuang *et al*., 1996; Ishitani *et al*., 1997; Ishitani and Chuang 1996; Ishitani *et al*., 1996a; Ishitani *et al*., 1996b; Saunders *et al*., 1999). Assigning multiple functions to the same protein is not only energy efficient to cells, as it requires making fewer proteins for various functions, but also implies that different cellular activities can be coordinately regulated (Jeffrey, 1999). In the case of GAPDH, it potentiates the coordination of glucose metabolism with other processes, including apoptosis, which (in yeast, at least) is a

mode of cell death that can be activated by oxidative stress – metal induced, or otherwise (Madeo, 2002; Nargund, 2008).

Cells employ sensor proteins that are able to ascertain the metabolic state of the cell and translate those to stress responses. The assignation of multiple functions to glycolytic enzymes such as GAPDH allows the metabolic enzyme itself to be efficiently used as a metabolic sensor and stress mediator. Indeed, the oxidization, or other (additional) post-translational modifications of this central glycolytic enzyme have been implicated as being a requisite step in the induction of apoptosis, one of the more drastic cellular responses to oxidative stress in higher eukaryotes (Hara *et al*., 2005; Cahuana *et al*., 2004).

While *Saccharomyces cerevisiae* adopts a number of the more critical steps in apoptotic programmed cell death that have been found in many higher eukaryotes, it lacks a number of the crucial regulatory proteins that have been found in higher organisms (Madeo, 2002; Madeo, 2004). Moreover, the presence of only a single metacaspase (Yca1) in yeast would suggest that the apoptotic process in this unicellular organism is considerably less convoluted than it is in higher organisms. Consequently, any requirement for GAPDH to function, as it does in higher organisms (Hara *et al*., 2005; Cahuana *et al*., 2004), as part of an apoptotic response in yeast needs to be characterized further. Therefore at the outset, therefore, of this investigation in to the role of GAPDH in yeast, we tested yeast cells that had been deleted for *tdh3* (the major isogene of GAPDH) for their ability to undergo apoptosis. Unlike wild-type cells with an intact GAPDH gene, the *tdh3*Δ strain of yeast was unable to undergo apoptosis in the presence of cadmium (Nargund, Shanmuganathan and Houghton, unpublished observations), which would indicate

that GAPDH is indeed required for apoptosis in yeast. It is pertinent to note that in yeast, there are three isoenzymes of GAPDH, Tdh1, Tdh2 and Tdh3 of which Tdh3 contributes the majority of GAPDH. Yeasts with mutations inactivating *tdh3* have been shown to retain viability that grew at rates equivalent to 75% of wild-type cells (McAllister and Holland, 1985). Since GAPDH is absolutely essential for growth in glucose, this shows that even in the absence of Tdh3 cells are able to metabolize glucose, albeit at a slower rate. Consequently, while the importance of the degree of glycolytic activity on apoptosis in yeast still needs to be addressed further, the finding that *tdh3* mutants are not deficient in glycolytic activity strongly suggests that the absence of apoptosis in *tdh3* mutants is not due to any deficiency in glycolysis itself, but rather it is more likely to be due to a specific loss of Tdh3 activity that is not necessarily related to glycolysis.

When GAPDH takes part in apoptosis, an increase in GAPDH levels has been reported to occur (Ishitani *et al*., 1996a; Ishitani *et al*., 1996b; Sawa *et al*., 1997). Ishitani *et al*., (1996b) have observed that transcriptional increases underlie the increase in protein. This pattern is so often seen that it can be referred to as a hallmark of apoptosis. Here too we report an increase in GAPDH transcripts and protein after an hour of exposure to cadmium, preceding the onset of apoptosis. However, yeast cells deleted for the caspase gene *yca1*, which fail to undergo apoptosis, also exhibit an increase in GAPDH protein levels (Figure 33D). There was no change in the relative fold change between wild-type and *yca1*Δ yeast, indicating that the changes in GAPDH precede and lie upstream of the caspase involvement in yeast apoptosis.

For GAPDH to participate in apoptosis, one of the key events is that the protein translocates to the nucleus (Dastoor *et al*., 2001; Sawa *et al*., 1997; Ishitani *et al*., 1999a and Hara *et al*, 2005) from its traditional location in the cytoplasm. As a next step in characterizing GAPDH in relation to apoptosis, therefore, we analyzed the cytosol and the nucleus of yeast for the presence of GAPDH before and after the apoptotic stimulus (cadmium exposure). We observed the cytosolic GAPDH to increase by 2 fold following metal exposure. Opinion is varied about the status and abundance of GAPDH in the cytosol subsequent to apoptosis. While some groups report a decrease in cytosolic GAPDH (Saunders *et al*., 1998) during nuclear translocation, others have in fact seen the cytosolic GAPDH to increase in rat hepatocytes undergoing apoptosis (Barbini *et al*., 2007) or remain unchanged in retinal muller cells undergoing apoptosis (Kusner *et al*., 2004). Accompanying an increase in the levels of GAPDH in the cytosol subsequent to metal exposure, the nuclear fractions exhibited a five-fold increased presence of GAPDH. This exaggerated fold change in the Cd treated nuclei, surpassing the fold-change in the cytosol, is indicative of nuclear translocation of GAPDH in spite of the cytosolic contamination seen in the nuclear extracts. A very pertinent question here is - how does GAPDH gain access to the nucleus? Under conditions of nitrosative stress in neuronal cells, GAPDH has been found to be S-nitrosated and by that virtue binds to another protein, SiaH. SiaH is stabilized by binding to GAPDH and in turn provides the nuclear translocation signal for GAPDH to enter the nucleus (Hara *et al*., 2005 and Hara *et al*., 2006). But there is no SiaH homologue in yeast and therefore the mechanism of nuclear translocation of GAPDH is still unclear. Moreover, the authors have also shown that under oxidative stress GAPDH does not bind to SiaH efficiently. Thus, it is conceivable that, in yeast, the same protein employs different strategies to gain access to the nucleus depending on the source and nature of the stress.

Previous work (Saunders *et al*, 1998 and Sunderaraj *et al*., 2004) has demonstrated that the nuclear GAPDH ($GAPDH_N$) is a distinct form with a different isoelectric point compared to the cytosolic form of GAPDH ($GAPDH_C$). There is little consensus on this issue, however, which is compounded by the paucity of investigations that have actually delved into the isoformic nature of GAPDH. While Saunders *et al*., (1998) have found that the $GAPDH_N$ has a lower pI and is more acidic, Sunderaraj *et al*., (2004) have reported that the $GAPDH_N$ exhibits a considerably greater pI and is rather basic. Our isoelectric point analysis of GAPDH shows that after Cd exposure, there is a shift in pI towards the basic end with slightly smaller forms of GAPDH being upregulated after Cd exposure. Even so, we did not see any considerable distinction in the pI of the $GAPDH_N$ and $GAPDH_C$ forms.

We also observed that Cd appears to induce fragmentation of GAPDH into smaller molecular weight forms lower in molecular weight by 5-10KD. Different functions have in the past been attributed to alternately spliced forms of proteins. An alternately spliced form of enolase, to cite an example, is involved in gene transcription (Subramanian and Miller, 2000). We were unable to determine whether these smaller GAPDH fragments are passive by-products of excessive oxidation of the protein, an alternately spliced form or a proteolytically cleaved product of GAPDH. We did verify, however, that these fragments are not formed as a result of caspase mediated proteolysis, as they continued to be present in yeast cells deleted for the caspase (*yca1*Δ). Interestingly, these fragments were present in the nuclear fraction and absent in the cytosol, which adds an interesting dimension to their relevance giving room for the speculation that these fragments may have a discretely nuclear function.

Aggregated GAPDH has been shown in Alzheimer's disease brains to accumulate in the cell, especially in the nucleus, and trigger cell death (Cumming *et al*., 2005 and Nakajima *et al*., 2007). Since Alzheimer's disease also involves oxidative stress mechanisms in a manner similar to metal toxicity, we sought to determine if indeed GAPDH aggregates in response to metal exposure. We prepared aggregated or insoluble protein fractions and observed that GAPDH levels increased in the aggregated fraction (Figure 35) subsequent to 3 hours of metal exposure. In order to determine whether disulfide linkages mediated this protein aggregation, we analyzed GAPDH under non-reducing conditions, when disulfide interactions between proteins are maintained. We observed that GAPDH maintains higher order structures and complexes before metal exposure and loses these disulfide-mediated interactions with itself and / or other proteins subsequent to cadmium exposure. Diagonal gel electrophoresis further confirmed these bands to be GAPDH owing to their position along the molecular weight of 35 KDa, similar to monomeric GAPDH.

Taken together, our results show that GAPDH is upregulated and modified by oxidation after cadmium exposure. Subsequent to this, GAPDH translocates to the nucleus, although the exact mechanism of this process is still unknown. Owing to its oxidation, fragmentation as well as translocation, GAPDH undergoes significant modifications, which result in the loss of many protein-protein interactions maintained by GAPDH before cadmium exposure. This work has thus characterized the many changes that GAPH undergoes upon cadmium exposure in an effort to understand how it is involved in apoptosis.

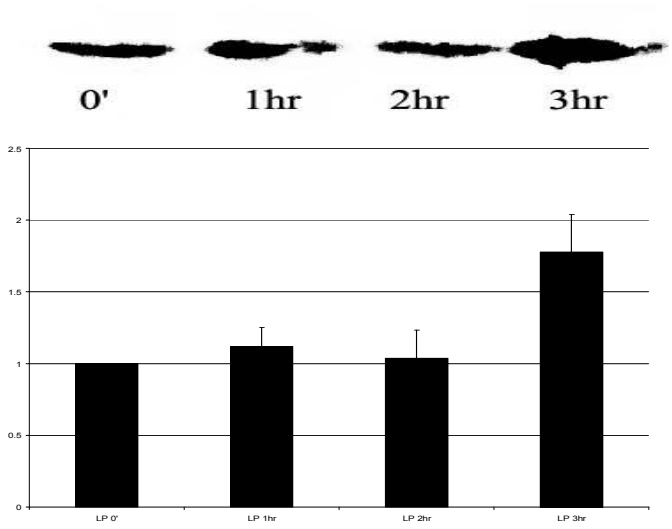

Figure 35. GAPDH increasingly aggregates in response to cadmium exposure

Yeast cells were harvested before and after cadmium exposure. Harvested yeast cells were lysed using a bead beater and centrifuged to separate the soluble proteins in the supernatant from the aggregated proteins in the insoluble pellet. Aggregated proteins were extracted from this insoluble pellet and analyzed by Western blotting for the presence of GAPDH.

Acknowledgements

This research was supported by grants from the National Institutes of Health (GM579450), the Georgia Research Alliance and Georgia State University (RPE awards) and by the Molecular Basis of Disease Fellowship awarded to A. S

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An Analysis of Glycolytic Enzymes in the Cellular Response to Metal Toxicity

General Discussion

This study has characterized the immediate protein oxidation and damage incurred by yeast cells as a consequence of heavy metal toxicity, brought about by exposure of these cells to low (effectively sub-lethal) concentrations of copper, chromium and cadmium. Although protein oxidation was previously known to occur as a result of such metal exposure, the specific targets and the consequences of metal-induced oxidative stress had not been well characterized. We have successfully identified glycolytic proteins, heat-shock proteins and actin to be the major proteins targeted for oxidation and,having also addressed some of the metabolic consequences of such targeted oxidation, argued that protein oxidation is one of the singular most important immediate signals for cellular oxidative damage, above and beyond the cellular cues derived from DNA and lipid oxidation. These results fill a scientific void that exists in the field of metal toxicity and its associated cellular damage. The findings are significant due to increasing evidence implicating protein damage as the principle mechanism of metal toxicity (Ding et al., 2006; Levine et al., 2000; Sumner et al., 2005). These results are all the more important as oxidative protein damage is held responsible for the pathogenesis of over 50 human diseases (Requena et al., 2001).

By choosing a sub-lethal dose of various metals, unlike other studies before us that have used highly lethal doses or LD_{50} (Vido *et al*., 2001 and Fauchon *et al*., 2002), we were able to ensure that the proteins under study were a representative sample of all cells, and that the various protein oxidation and abundance profiles were not varied as a function of a mixture of live and dead cells in the culture. Also, unlike many other studies that have dealt with long-term effects, we have studied the responses occurring at the level of the proteome within an hour of metal exposure, since previous work by our associates had (Avery *et al*., 2000) established that protein oxidation (due to metal exposure) was maximal within the first hour. This time frame is one of the distinguishing parameters of this series of investigations and has proven crucial to our understanding of the initial, first-line of responses to metal exposure, which decide the cellular fate thereafter. Since metals vary in their mechanism of toxicity, we have chosen three metals that are representative of the action of all metals. Among the metals under investigation, Cu and Cr are redox-active and can generate ROS directly whereas Cd is redox-inactive and generates ROS by replacing redox-active metals from the active site of enzymes (Wei et al., 2001), by depleting sulfhydryls (Stohs and Bagchi, 1995) or by mitochondrial perturbation (Pacheco *et al*., 2007). Cr is distinct among this group, since it can undergo redox-cycling among its various ionic forms and produce a variety of ROS. In response to prooxidant stressors, there are overlaps as well as differences (Gasch and Werner-Washburne, 2002; Gross *et al*., 2000; Lee *et al*., 1999; van Bakel *et al*., 2005). The response to Cd is known to invoke the transcriptional factor Yap1 (Vido *et al*., 2001; Wemmie *et al*., 1994; Wu *et al*., 1993) in a manner similar to hydrogen peroxide and thus the response to Cd has long been thought to be a peroxide-type response. The response to Cu does not involve Yap1, but instead invokes Ace1 and the metallothioneins

(Slekar et al., 1996). The copper response is therefore considered to be quite distinct from the cadmium response, although it was interesting to note from our analyses the number of similarities in the proteomic responses to Cu and Cd *viz.* protein expression and protein oxidation.

In spite of the differences in redox chemistry and the mode of toxicity, the yeast cells respond not only to protein expression but also to protein oxidation in a very similar manner to each of these three metals. Consistent with the previous observations that protein carbonyl content peaks within one hour of metal exposure (Avery *et al*., 2000), we too observed an increase in protein carbonyl content within as early as 5 minutes of metal exposure (Figures 5, 12 and 14). Carbonyl content continued to increase, reaching a maximum after 15-30' of metal exposure and decreasing thereafter over the course of one hour. This decrease can be explained by the potential intervention of antioxidant defenses (enzymatic and non-enzymatic). Indeed, through our proteomic analyses, we have observed a two-fold increase in the levels of the cytosolic Cu/Zn superoxide dismutase (Sod1) in response to Cu, Cd and Cr within the first hour of metal exposure (Chapter 4) as well as a three-fold increase in the levels of reduced glutathione (GSH) in response to Cd exposure (Figure 19). The similarities in protein oxidation extend beyond the temporal profiles to the oxidation targets themselves. The protein oxidation targets are glycolytic enzymes, proteins involved in glucose metabolism, heat shock proteins and actin. A majority of these proteins are also targeted for oxidation (Cabiscol *et al*., 2000; Costa *et al*., 2002) and S-thiolation (Shenton and Grant, 2003) in response to hydrogen peroxide and menadione induced oxidative stress. This suggests that damage to these proteins is a conserved response and is independent of the type or the mechanism of action of the oxidant stressor.

It is worth noting that a number of highly oxidized proteins are also highly expressed in the cell. We therefore initially considered it possible that proteins are oxidized based upon abundance. However, this thought does not stand up to the observation that proteins with similar abundance are targeted differently for oxidation (Figure 15 E-F). In truth, even isoforms of the same protein are targeted differently and in a number of cases (Figure 15G-J and Figure 4) it is the less abundant isoform which is more heavily oxidized. These observations suggest that oxidation is very much a targeted phenomenon, which we have argued is based upon structure and/or location and/or function and not abundance of the protein. In the following section, we discuss the potential reason(s) behind why these proteins are susceptible to oxidation.

Let us first consider structural susceptibility of these proteins. It is interesting to note that all proteins that are oxidized in response to metals contain Cys residues that are among the most oxidation susceptible amino acids. By comparing the amino acid sequence of the proteins, we observed that the targeted proteins are cysteine rich. However, with the exception of enolase (Eno1 and Eno2) and glyceraldehyde phosphate dehydrogenase (Tdh3), the Cys residues are not in the active site of these proteins. It is intriguing therefore that these susceptible residues are rigidly conserved in these highly evolved ancient enzymes. Why retain the weak link? It may not be unwise to speculate that these residues have been retained to precisely promote a targeted, immediate oxidation response to various oxidant stressors so as to reroute metabolism towards the PPP, which offers an adaptive response towards oxidative stress.

It is conceivable that these proteins could be rendered sensitive to oxidation based upon their location in the cell. Recent proteomic analyses on mitochondrial proteins in yeast demonstrated that a number of glycolytically related enzymes that are oxidation targets are either attached to the mitochondrial membrane or present within the mitochondrion itself (Ohlmeier et al., 2004; Reinders and Sickmann, 2007). Such a location for the glycolytic enzymes would enable a more efficient transport of glycolytic end products into the mitochondria where they will be further metabolized by the members of the TCA cycle. As mitochondria are considered to be the primary oxidant source in the eukaryotic cell, the proximity or preferential association of cytosolic enzymes to the outer membrane of the mitochondria may hold some clue as to why specific proteins and enzymes are more susceptible to oxidation. While we did not specifically address the mitochondrial association of the oxidized proteins in this report, it is of interest that only some of the isoforms of glycolytically related enzymes that were found to be oxidized in response to Cd were clearly shown to be associated with the mitochondria (Ohlmeier *et al*., 2004). Furthermore, glycolytic enzymes such as aldolase and enolase are also known to physically interact with members of the cytoskeleton, particularly actin (Arnold and Pette, 1970; Pancholi, 2001), which is an established oxidation target (Haarer and Amberg, 2004). Proximity to sources of ROS (mitochondria) as well as proximity to the highly oxidized actin potentially could be a reason why the glycolytic proteins are major targets.

We have considered the possibility that the susceptibility to oxidation is based upon protein structure and/ or location in the cell; however it is also quite possible that these proteins are specifically targeted based upon their function. Let us consider the correlation of susceptibility to the function of the major groups of targets – the heat shock proteins, and the

glycolytic enzymes. HSPs are chaperones that function by assisting in protein folding. HSPs are therefore considered to closely interact with mildly oxidized and consequently misfolded proteins. Proximity to damaged proteins could render HSPs to be damaged i.e., the close interaction between HSPs and other oxidized proteins would mediate a protein-protein radical transfer resulting in HSP oxidation (Magi *et al*., 2004). Thus, HSPs are thought to act as shields for other proteins and in the process get oxidized themselves (Cabiscol et al., 2000). We have observed HSPs to be oxidized by all three metals. This is not to say that all three metals elicit identical patterns of HSP oxidation. Comparing Cd, Cu and Cr induced oxidation profiles, although some HSPs (such as Hsp60 and Ssa1/Hsp70) are increasingly oxidized over time in Cd treated cells (Figure 36H), the onset and degree of their oxidation being far less than the corresponding oxidation profiles in either Cu (Figure 36D) or Cr stressed cells (Figure 36F). Moreover, these differences in oxidation levels occur with no discernable change in their relative abundance (Figure 36 panels A, C, E and G). Changes in HSP oxidation patterns, therefore, may reflect significant mechanistic differences in metal toxicity. Among the three metals studied, Cr exerts the highest damage to HSPs, with a number of additional cytoplasmic and mitochondrial chaperones (Ssb1/Hsp75, Ssc1/mtHsp70) can be clearly identified as being oxidized (Figure 36F). This would imply that Cr elicits a greater degree of protein damage, an implication that is entirely consistent with the experimental findings of our collaborators in Nottingham, UK, who found that extensive protein aggregation resulted from Cr exposure (McAllister and Holland, 1985), a toxic effect that has been shown to be exerted primarily through protein damage (Grune and Davies, 1997; Shrivastava and Nair, 2000; Sumner *et al*., 2005). Hsp60 and proteins of the Hsp70 family are also ATP binding proteins, and ATP has a high affinity for divalent metal ions. So, HSPs may be acting as a sink to metals and as a consequence getting oxidized

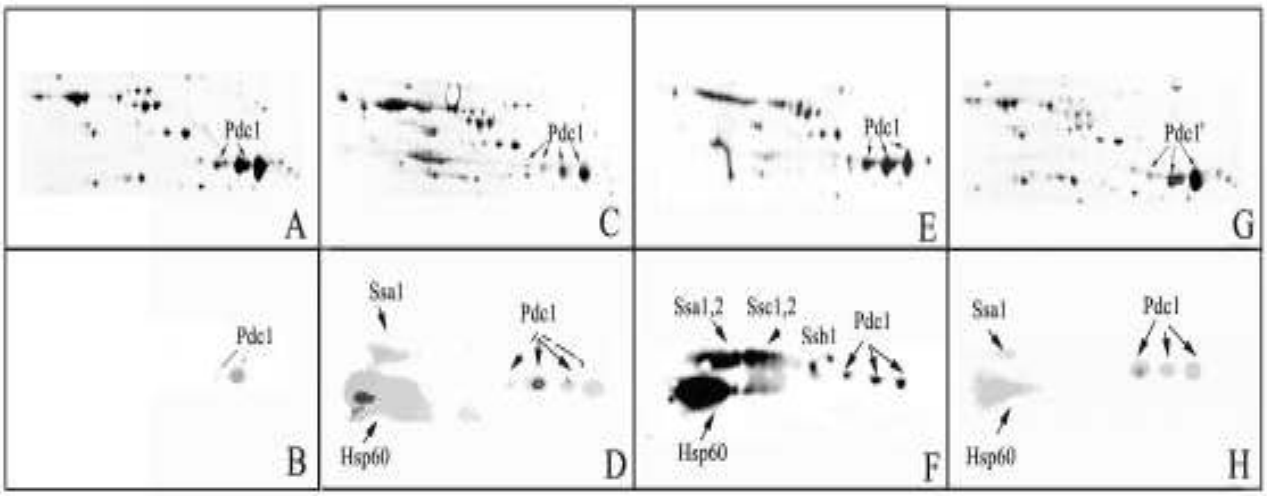

Figure 36. Oxidation of HSPs in response to Cu, Cr and Cd

Proteins were extracted from yeast cells before and after various time intervals up to 60 minutes after metal exposure. 2D gels prepared from these proteins were either stained with SYPRO Ruby (top panels) or the corresponding Western blots (bottom panels) were probed with anti-DNP antibodies for oxidized proteins. Panels A and B are from cells unexposed to metal, panels C and D are derived from cells after 60' of 8mM Cu $(NO_3)_2$ exposure, panels E and F are derived from cells after 60' of 0.5mM CrO_3 exposure and panels G and H are derived from cells after 60' of 30 μM $Cd(NO_3)_2$ exposure. Location of Pdc1 isoforms can be used as reference spots. Cu and Cr data are derived from previously published materials *(*Shanmuganathan, 2004*)* and *(*Sumner, 2005*)*, respectively. Gels and blots are representative of representative of two gel separations undertaken after different times of Cd exposure.

(Cabiscol et al., 2000). Oxidation of the chaperones themselves leads to further protein damage, protein misfolding and aggregation all of which are detrimental to the cell.

The other group of oxidized proteins that have been defined in the series of investigations, detailed in this dissertation, includes enzymes that exist as 3 or more isoforms and are involved in glycolysis or further metabolism of glucose. Seeing such a specific and targeted response towards the glycolytic proteins raises the question of metabolic consequences of such a targeting. Since it is established that protein oxidation compromises the function of the protein (Shacter, 2000), we hypothesized that glycolytic protein oxidation would result in glycolytic inhibition and promote flux through the alternative glucose-metabolizing pathway, the pentose phosphate pathway. Increased flux through the pentose phosphate pathway has been shown to improve resistance to oxidative stress (Boada *et al*., 2000). We therefore hypothesized that the glycolytic protein oxidation would result in a transient reduction in glycolytic flux, which would achieve an increased flux through the PPP and thereby promote resistance to oxidative stress. The PPP is the major source of cellular reducing power ($NADPH_2$), which is crucial in maintaining the redox status of the cell, especially in an oxidatively stressed condition. Not only is reducing power needed for redox equilibrium, it is also required as a cofactor by a number of antioxidant enzymes, including glutathione reductase and thioredoxin reductase which recycle the oxidized forms of glutathione and thioredoxin, respectively, to the reduced forms.

To assess the potential metabolic consequences of such changes in glycolytic flow, we first assessed the effects of Cu, Cd and Cr on the activities of some of the key enzymes of the glycolytic pathway. Cu was the most insidious, causing a decrease in the activity of alcohol dehydrogenase by 60% (Figure 37A), of glyceraldehyde 3-phosphate dehydrogenase by 40% (Figure 37B) and of enolase by 45% (Figure 38). The maximum reduction in enzyme activity

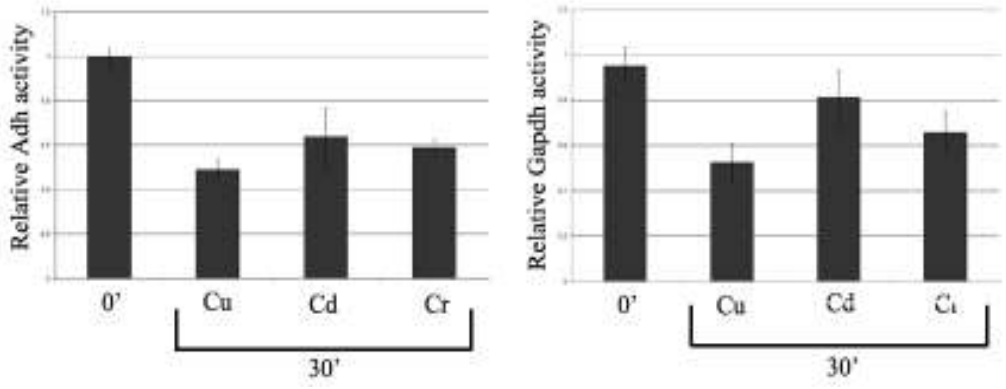

Figure 37. Alcohol dehydrogenase and glyceraldehyde 3-phosphate activities are reduced after metal exposure

Yeast cells were analyzed for Adh and Gapdh activities before and after 30' of 8mM Cu or 30 uMCd or 0.5 mM Cr exposure. Activities were normalized to protein concentration and depicted relative to control

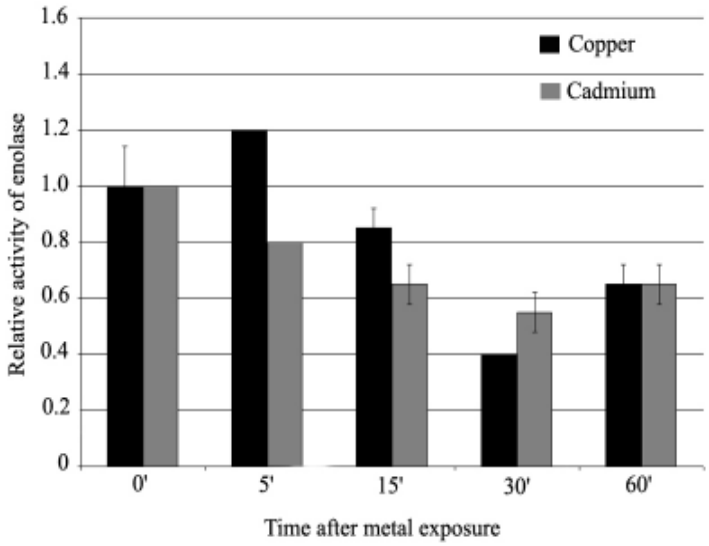

Figure 38. Enolase activity reduces after metal exposure

Yeast cells were analyzed for enolase activity before and after varying times of 8mM Cu (black bars) or 30 μM Cd (grey bars) exposure. Activities were normalized to protein concentration and depicted relative to control

coincided with the peak in oxidation levels, which was around 15-30' after Cu exposure. Cr and Cd exerted similar effects on the activities of these enzymes, which were decreased by 20% for alcohol dehydrogenase (Figure 37A), 40% for glyceraldehyde 3-phosphate dehydrogenase (Figure 37B) and by 45% for Enolase (Figure 38). Such a coordinate and concerted inactivation of these critical enzymes would strongly indicate that the glycolytic pathway itself is severely compromised in response to all three metals, leading to an overall reduction in the flow through the pathway, which was in line with our original hypothesis.

Having seen a reduction in glycolysis, we analyzed the expression of genes encoding the enzymes constituting the pentose phosphate pathway (PPP), which provides the alternate route to glycolysis for carbon metabolism. We observed not only a transcriptional induction in key enzymes, including Zwf1 (Figure 17), but also a gradual, albeit modest, increase in Zwf1 enzyme activity (Figure 17). The glycolytic inhibition together with PPP induction is indicative of an increased carbon flow through the PPP. This was confirmed by an increase in the levels of $NADPH_2$ for both Cd (Figure 18) and Cu. In cells exposed to cadmium there was there was a 3-fold increase in the levels of reduced glutathione (GSH) that was concomitant with the increase in $NADPH_2$ (Figure 19A), and which was decidedly absent in cells that had been similarly exposed to Cu (Figure 19A inset). GSH is a known player in Cd detoxification (Li *et al*., 1997; Smith *et al*., 2007). Apart from its established role as an antioxidant, GSH also directly binds to Cd, which results in vacuolar sequestration thereby aiding Cd removal (Li *et al*., 1997; Vido *et al*., 2001). Therefore, it is not surprising that the increase in PPP is reflected by an increase in GSH only for Cd and not for Cu. It is conceivable that the $NADPH_2$ generated in response to Cu

is utilized for non-glutathione purposes, such as to restore the redox equilibrium in the cell or increase reduction capacity in the cell through reduction of the thioredoxin network. It should also be noted that the increase in levels of GSH in response to Cd is not as pronounced in mutants of the pentose phosphate pathway (*zwf1*Δ and *gnd1*Δ) (Figure 19A and Figure 39) demonstrating the importance of PPP for glutathione production.

We have proposed that the glycolytic proteins are targeted for oxidation to achieve metabolic reshuffling to the PPP. While it is true that both Cu and Cd inhibit glycolysis and induce PPP, Cu fails to use the resulting reducing power to increase the levels of GSH. This indicates that glutathione production may not be the only consequence of the susceptibility of these proteins to oxidation. Moreover, glycolysis and, more specifically, certain glycolytic proteins are involved in a number of additional pleiotropic functions, some of which include a mediating role in programmed cell death, including apoptosis (Kim and Dang, 2005; Sriram 2005 and Table 7). Glycolytic inhibition and the consequential modulation to cellular metabolism are documented in several apoptotic studies (England *et al*., 2004; Gonin-Geraud *et al*., 2002 and Otsuki *et al*., 2005). It is also being discovered that oxidized forms of specific proteins may serve as signals of cell death. Actin, HSPs and some of the glycolytic enzymes are now being seen in an entirely new light as inducers of apoptosis. Actin, which forms the cytoskeleton of the cell is oxidized in response to both Cu and Cd. Oxidation of actin modifies its phenotype by increasing actin filament assembly, hyperstabilizing actin filaments and increasing sensitivity to oxidative stress (Haarer and Amberg, 2004). A model has been proposed wherein oxidized, hyperstabilized actin alters mitochondrial stability, leading to a leaking of ROS from the mitochondria which results in further oxidation of actin, establishing a positive feedback loop

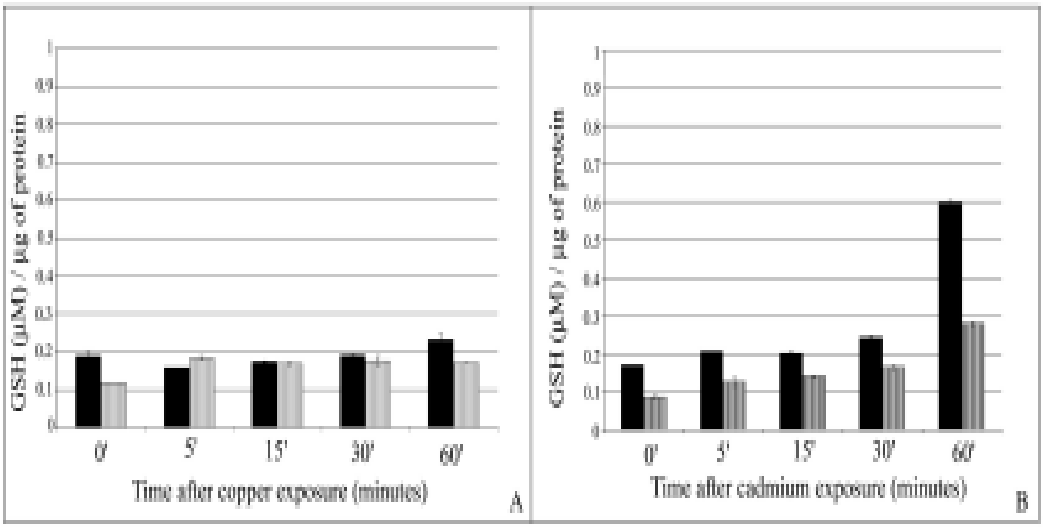

Figure 39. Reduced glutathione levels in Wild-Type yeast cells and *gnd1*Δ yeast cells

Cell extracts were prepared from wild-type and gnd1□ yeast cells before and after exposure to 8mM $Cu(NO_3)_2$ (A) or 30 μM $Cd(NO_3)_2$ (B), and GSH levels determined in cell extracts (see Chapter 3 - Materials and Methods). Reduced GSH levels were normalized to protein concentrations of the extracts. The data shown are representative of three independent assays. Wild-Type GSH levels are depicted as solid black bars and *gnd1*Δ GSH levels are depicted as patterned grey bars.

leading to cell death (Farah and Amberg, 2007). Because mitochondrial instability is often associated with apoptosis, actin has been advocated to be the primary sensor of oxidative stress. HSP oxidation is also pertinent to apoptosis.

As we have discussed before, oxidation of HSPs leads to an accumulation of misfolded and aggregated proteins, which is a known trigger for apoptosis. Moreover, glycolytic enzymes are functional in non-glycolytic arenas and are being regarded as multi-functional and pleiotropic enzymes. For example, enolase has been found to be present in the nucleus and to take part in transcriptional regulation (Subramanian and Miller, 2000). Another glycolytic protein, hexokinase, is considered to be anti-apoptotic (Abu-Hamad *et al*., 2008). Yet another protein with pleiotropic function is glyceraldehyde 3-phosphate dehydrogenase, which plays a role in DNA repair (Krynetski *et al*., 2001), regulating telomere length and controlling cell senescence (Sundararaj *et al*., 2004) and is also considered to be essential in neuronal apoptosis (Ishitani *et al*., 1996 and Hara *et al*., 2005). Since a number of these functions are ascribed to oxidized forms of the proteins it is plausible that the oxidative susceptibility of these proteins is based upon their role as apoptotic initiators i.e. oxidized proteins may well be apoptotic signals. Oxidized GAPDH has already been implicated in apoptosis and heralded a "sensor of oxidative stress" (Cahuana *et al*., 2004). In the preceding chapter, the role and effects of the pro-apoptotic GAPDH in cadmium toxicity in yeast were addressed.

In these series of analyses, we were able to verify that GAPDH was required for apoptosis in yeast and that it was oxidized (carbonylated) during cadmium exposure. Thereafter, we proceeded to characterize the changes in GAPDH in order to understand its role in apoptosis. Subsequent to cadmium exposure, the levels of GAPDH were upregulated at the transcriptional

level. This has often been observed in the past and is therefore considered to be an important indicator of GAPDH's involvement in apoptosis. The transcriptional increase in the *tdh* genes, particularly in *tdh3*, was accompanied by corresponding increases at the protein level. Moreover, we were also able to demonstrate that the increase in GAPDH levels in the whole cell resulted in an enhanced presence of GAPDH in the nucleus. The translocation of GAPDH that was seen to occur subsequent to cadmium exposure was isoform specific. Moreover, following cadmium exposure, GAPDH increasingly appeared to exist as smaller molecular weight fragments of the GAPDH protein, which were concentrated in the nuclei and absent in the cytoplasm. As a result of many modifications such as oxidation, fragmentation and translocations to a new sub-cellular location, GAPDH was found to lose the protein-protein interactions that were maintained, via disulfide linkages, prior to cadmium exposure. Even after some preliminary characterizations of these changes, several questions remain. In particular, how does GAPDH enter the nucleus and what is its role in the apoptotic cascade? Unfortunately, the answers to these questions, pertinent as they may be to this work, are beyond the scope of this dissertation.

This study has characterized protein oxidation and damage suffered by yeast cells during metal toxicity. Although protein oxidation was known to occur as a result of metal exposure, the specific targets and the consequences of oxidation were uncharacterized. We have successfully identified glycolytic proteins, heat-shock proteins and actin to be major oxidation targets and have also addressed the metabolic consequences of such targeted oxidation. These results fill the void that existed in the field of metal toxicity and its associated protein damage. The findings are significant due to increasing evidence implicating protein damage as the principle mechanism of metal toxicity (Ding et al., 2006; Levine et al., 2000; Sumner et al., 2005). These results are all

the more important as oxidative protein damage is held responsible for the pathogenesis of over 50 diseases (Requena et al., 2001).

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