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 hypersensitive to Cr(VI) toxicity under aerobic conditions, but this phenotype was suppressed under anaerobic conditions. Studies with cells expressing a Sod1p variant (Sod1pH46C) 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 the 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 micro-organisms, and metal exposure of higher organisms is associated with a range of harmful effects; for example, Cr exposure has been linked to genotoxicity, carcinogenicity and allergenicity (Dayan & 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 Cr at the cellular or molecular level and this needs to be addressed.

Cr 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 hypothesized 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 catalyse Fenton-type reactions to promote free radical formation (Halliwell & 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 micro-organisms is of interest in its own right, from both environmental and biotechnological perspectives (White et al., 1998; Cervantes et al., 2001).

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 (Cervantes et al., 1999; O’Brien et al., 2001; Ackerley et al., 2004). 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; O’Brien et al., 2002; 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) (O’Brien et al., 2002). Protection against Cr by ROS-scavenging molecules and other antioxidants has been reported in several organisms (Pourahmad & O’Brien, 2001; Pestil et al., 2002). In S. cerevisiae, Cr has been shown to affect mitochondrial function (Henderson, 1989; Fernandes et al., 2002), and the antioxidant protein alkyl hydroperoxide reductase (Ahp1p) protects against Cr toxicity (Nguyen-Nhu & Knoop, 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.

**METHODS**

**Strains and plasmids.** Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and derivative deletion mutants sod1Δ, gpx1Δ, ogg1Δ, msrαΔ (msr1Δ) and msrβΔ (ycl033ΔA) were obtained from Eurocarb. An isogenic gpx1Δ/gpx2Δ/gpx3Δ triple mutant was constructed previously (Avery & Avery, 2001). An msrαΔ/msrβΔ 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/rad3Δα (Harasca et al., 2000) were gifts from L. Prakash, University of Texas. The plasmids YEp351-Sod1 (Nishida et al., 1994) and YEp351-Sod1H46C (Lu et al., 1996; Wei et al., 2001) were kindly provided by E. B. Gralla (University of California). All plasmids were transformed into S. cerevisiae according to Gietz & Woods (2002). Plasmid YEp352-MsrB was constructed in this study after PCR amplification from yeast genomic DNA of a fragment comprising the full MSRB ORF together with 600 bp of upstream non-coding sequence (primary sequences are available on request). The amplified fragment was ligated between the SacI and KpnI 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 30°C, 120 r.p.m., to mid-/late-exponential phase (OD600 ~ 2–0) either in liquid YEPD or in YNB medium for experiments involving plasmid-bearing strains. For enumeration of viable cells on CrO3-supplemented agar, the experimental cultures were adjusted to an OD600 of ~0.004 in sterile YEPD broth and 200 μl aliquots were plated onto YEPD agar supplemented with CrO3 at the desired concentration. Colony-forming ability was determined after 8 d at 30°C.

For spotting experiments, experimental cultures were each adjusted to an OD600 of ~2–5, 0.25, 0.025, 0.0025 and 0.00025. Samples (4 μl) from each dilution were spotted onto YEPD agar, supplemented with CrO3 as specified. Growth was examined after incubation for 4 d at 30°C. Where specified, plates were incubated anaerobically in an N2 atmosphere. For experiments involving linolenic acid (18:3), media were prepared with tergitol (Nonidet P-40; Sigma) to solubilize the fatty acid and 1 μl aliquots were spotted from dilution series starting at an OD600 of ~5–0. The final tergitol concentration was 1% (w/v); tergitol has no adverse effect on yeast growth (Howlett & Avery, 1997).

**Analysis of protein oxidation.** At intervals during exposure to 0.5 mM CrO3 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 essentially as described by 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 carboxyls 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.; Amersham Pharmacia (or pH 4–9) to 1:16 000 dilution) and peroxidase-linked goat anti-rabbit IgG as secondary antibody (Sigma; 1:16 000 dilution). Carboxylated proteins were immunodetected with a chemiluminescent peroxidase substrate, West femtoM (Pierce), using a Fuji LAS3000 Image Analyser with pre-cooled camera. Quantification of carboxylated 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 database www.ibgc.u-bordeaux2.fr/YPM and www.expasy.org/images/swiss-2dpage/publi/yeast-high.gif. The identities of specified proteins within the protein arrays were subsequently confirmed by MALDI-MS (Voyager DE Pro; Applied Biosystems) as described previously (Shanmuganathan et al., 2004).

**RESULTS**

**Cr 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; note that Cr(VI) (as CrO3) was the Cr species tested throughout this study, due to its high
toxicity. An *S. cerevisiae* sod1Δ deletion strain was found to be markedly more sensitive to Cr toxicity than wild-type cells (Fig. 1). Thus, the onset of Cr toxicity (loss of colony-forming ability) commenced at approximately 0.23 and 0.65 mM CrO$_3$ for the sod1Δ and wild-type cells, respectively, with approximately 0.5 and 1.1 mM Cr being required to give 90% loss of viability of these strains, respectively (Fig. 1).

Sod1p-dependent resistance to Cu in *S. cerevisiae* was found previously to involve Cu buffering rather than ROS scavenging by the enzyme (Culotta et al., 1995). There is evidence that Sod1p-binding of Zn (Wei et al., 2001) and of Ag (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 (Fig. 2a). 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 sod1Δ cells transformed with plasmids expressing either a wild-type SOD1 ORF or a mutant sod1 allele encoding Sod1H46C. Sod1H46C lacks superoxide dismutase catalytic activity, but is minimally affected for metal-binding capacity (Lu et al., 1996; Wei et al., 2001). The wild-type SOD1 gene complemented the sod1Δ phenotype, restoring growth in the presence of Cr to wild-type levels (Fig. 2b). In contrast, Sod1H46C had no influence on
the Cr sensitivity of the sod1Δ mutant even when expressed on a multi-copy plasmid [YEp351-Sod1146C], which has been characterized previously (Wei et al., 2001)]. Together, the results show that it is specifically the antioxidant function of Sod1p that protects S. cerevisiae from Cr toxicity.

Repair of protein oxidation is required for cellular Cr resistance, but not repair of DNA oxidation or lipid peroxidation

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 & Avery, 2001; Avery et al., 2004). Expression of Gpx3 confers resistance in yeast to agents which have a lipid peroxidation-dependent mode of action (Avery 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 gpx3Δ 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 gpx1Δ/gpx2Δ/gpx3Δ 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 linolenic acid [which is incorporated to greater than 60% of total membrane fatty acids under the conditions used (Avery et al., 1996; Howlett & Avery, 1997)] did not sensitize cells to Cr (not shown). Thus, in contrast to data from equivalent experiments performed with metals such as Cu and Cd (Avery et al., 1996, 2004; Howlett & Avery, 1997), 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 ogg1Δ cells were compared for Cr sensitivity, but no difference was apparent (data not shown). We obtained the same result also with an ogg1Δ/rad30Δ double mutant, which is susceptible to a synergistic increase in oxidative DNA lesions (Haraczka et al., 2000) and in pro-oxidant sensitivity (Willetts, 2004) versus the ogg1Δ and rad30Δ 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 (methionine-S-sulfoxide and methionine-R-sulfoxide, respectively) (Kryukov et al., 2002). MSRs provide the only characterized protein oxidation repair activity of cells. Single msraΔ and msrbΔ mutant strains exhibited marked sensitivity to Cr compared to wild-type cells, and this phenotype was accentuated in a double msraΔ/msrbΔ mutant (Fig. 3a, b). Thus, the colony-forming ability of msraΔ/msrbΔ cells was diminished by approximately three orders of magnitude at 0.8 mM CrO3, a concentration at which colony formation by wild-type cells was barely affected (Fig. 3b). 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(NO3)2 (10–16 mM) and Cd(NO3)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 (Avery 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 (Fig. 3c). In addition, overexpression of MSRA and MSRB suppressed the Cr sensitivity phenotype of sod1Δ cells (Fig. 3d), suggesting that the enhanced Cr toxicity seen in this mutant (Figs 1 and 2) 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. 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 CrO3, 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 Fig. 3). A non-lethal concentration of 0.5 mM CrO3 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 permeabilization and protein leakage from some cells but not others, yielding non-representative protein...
Cr caused rapid, but transient oxidation of total-soluble proteins (Fig. 4a; proteins with pI in the range of 3–10 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 Fig. 4b) corresponded well with standard profiles for S. cerevisiae available in 2D protein gel databases (see Methods). The identity of each protein of interest was validated by MALDI-MS. Cr-dependent protein oxidation was evident from the increased intensity and numbers of carbonylated proteins in immunoblots derived from extracts of Cr-treated cells (Fig. 4b). 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) (Fig. 4b, Fig. 5). 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.

Similar proteins 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 sublethal Cu concentrations (Shanmuganathan et al., 2004) were normalized against the equivalent data for Cr (see legend to Fig. 5). Thus, differences were apparent in the relative carbonylation of individual proteins during Cr and Cu exposure (Fig. 5). 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, though 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 (Lu et al., 1996; Wei et al., 2001) 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 hypersensitivity phenotype of sod1Δ cells. It can be inferred that Cr exposure generates superoxide at toxic levels.

Cell-cycle- and age-dependent fluctuations in Sod1p have been found to drive variation in the Cu 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 (Culotta et al., 1995) 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 & Avery, 2001; Avery 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, Cd (Avery 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) is important for Cr resistance. Consistent with this, enrichment of S. cerevisiae with an oxidation-sensitive polyunsaturated fatty acid did not sensitize the cells to Cr, unlike the outcome found previously for Cu and Cd (Avery et al., 1996; Howlett & Avery, 1997). 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 (O’Brien et al., 2002; 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 cross-links (O’Brien et al., 2002; 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 & 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 apnl1/radl1/ntg1/ntg2 mutant, such lesions were not sufficient to elicit a Cr(VI) hypersensitivity phenotype (O’Brien et al., 2002).

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 (Jamieson, 1998) 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; Avery et al., 2004; Willetts, 2004) – enabled us to discriminate between these groups as the candidate toxicity targets of Cr. It is emphasized 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 (Avery 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 (Kryukov et al., 2002; Koc et al., 2004). While MSR enzymes have narrow specificity (for oxidized Met), Met residues are especially susceptible to metal-catalysed oxidation in proteins (Kim et al., 2001). 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 (Shrivastava & Nair, 2000, 2004; Feng et al., 2003). 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 Cu maximally gives only an ~8-fold increase (Shanmuganathan et al., 2004), with the metals supplied at just sublethal 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 and 60 min exposure to Cr likely reflects selective degradation of oxidatively damaged proteins (Grune et al., 1997).

Whereas the total carbonylation induced by Cr was relatively high, Cr targeted a similar range of yeast proteins to Cu (Shanmuganathan et al., 2004) and other pro-oxidants (Cabisco 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, so promoting the production of glucose equivalents within the pentose phosphate pathway (Ravichandran et al., 1994; Costa et al., 2002; Shenton & Grant, 2003). Such rerouting 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 NADPH2 necessary for the function of certain antioxidant enzymes (Godon et al., 1998; Cabiscol et al., 2000). Furthermore, rerouting may alleviate glucose repression of antioxidant gene transcription (Moradas-Ferreira et al., 1996).

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 H2O2 further supports an oxidative mode of Cr toxicity. Despite the similar subsets of proteins targeted, individual proteins exhibited differing relative susceptibilities specifically to Cr- or Cu-mediated oxidation. The proteins examined (Fig. 5) were all primarily cytosolic, suggesting that differential targeting by Cr and Cu is unlikely to 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 to identify the specific protein target(s) of cellular Cr toxicity.

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