Examination of mechanism of action of chromium compounds on eukaryotic cells

Ph.D. thesis

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INTRODUCTION

Among the effects of increasing industrialization that grossly contaminates the environment is the discharge of heavy metals in effluents. Chromium compounds are some of the best- documented mutagens and carcinogens. Chromium exists in many oxidation states, of which only Cr(VI) and Cr(III) ions are stable under environmental conditions. Water containing more than 0.05 mg/l of Cr(VI) is considered to be toxic. The water-soluble chromate(VI) ion is biologically active; it can readily penetrate through biomembranes via the anion-exchange channels, but it is rapidly reduced intracellularly to the kinetically much more stable Cr(III). About 90% of cellular chromium has been demonstrated to be present as Cr(III) species. In vitro experiments have revealed that Cr(III) ions form many complexes with biologically relevant ligand molecules; this situation is involved in DNA crosslinking, DNA-protein cross-linking, DNA condensation and decreasing DNA replication fidelity. The reduction process itself may contribute to the mutagenicity and carcinogenicity of Cr(VI) by producing the hydroxyl radical (WOH).

When Cr(VI) enters a cell, it undergoes several processes: (i) reduction of Cr(VI), (ii) reaction with reactive oxygen species (ROS) that results in formation of the harmful hydroxyl radical ($^{\bullet}OH$), and (iii) neutralization by cellular constituents, including detoxifying enzymes and antioxidants. It is generally accepted that the cyto- and genotoxic effects of Cr(VI) compounds can be attributed to the harmful $^{\bullet}OH$ generated in a Fenton reaction between H_2O_2 and Cr(V). The Haber-Weiss reaction between $O_2^{\bullet-}$ + Cr(VI) is unlikely to play a significant role in the formation of Cr(V) and hence in the generation of $^{\bullet}OH$.

The Cr(VI) tolerance of yeasts seems to be proportional to their ability to take up sulfate from the environment. The Cr(VI) gradient between the two sides of the cell membrane is maintained by the metabolically active cells themselves, which continuously reduce the accumulated Cr(VI) to lower oxidation states in both enzymatic processes (involving flavoenzymes) and nonenzymatic processes (glutathione (GSH), NADPH and ascorbate). Among the low molecular mass reductants, GSH is widespread in yeasts and seems to be the most important agent participating in the reduction of Cr(VI). The *in vitro* reduction of Cr(VI) by GSH generates the glutathione derived thiyl radical (GS•) and Cr(V). Importantly, among the GSH-dependent enzymes, GR has been shown *in vitro* experiments to reduce Cr(VI) directly to Cr(V), using NADPH as co-substrate, and with the concomitant generation of superoxide. EPR studies have demonstrated that chromium in various oxidation states, but especially Cr(V) and Cr(IV), is able to react with H_2O_2 and produce the radical $^{\bullet}OH$

in Fenton-type reactions. This deleterious, cyto and genotoxic radical may also be generated in a Cr(VI)-mediated Haber-Weiss-type reaction, but the direct participation of $O_2^{\bullet -}$ in the generation of $^{\bullet}OH$ has been questioned. It is well known that $^{\bullet}OH$ causes very severe cell damage because there is no enzymatic protection against it. The non-enzymatic $^{\bullet}OH$ scavengers include ascorbate, α-tocopherol and carotenoids, none of which are produced by *S. pombe* cells. This yeast probably uses GSH and phytochelatins. The GSH and free radical metabolisms of fungi are rather complex and are influenced by numerous endogenous and exogenous factors. Accordingly, characterization of both the GSH-dependent and the GSH-independent pathways of the antioxidative defence system was inevitable in this case. The first line of defence against $O_2^{\bullet -}$ and H_2O_2-mediated injuries includes well-characterized antioxidant enzymes, including superoxide dismutases (SODs), peroxidases and catalase, which are also key elements in the adaptation to and cross-protection against various oxidative stressors. The activity of GSH-dependent detoxification processes relying on glutathione S-transferase (GST) was recorded because this enzyme is crucial when superfluous glutathione disulfide (GSSG) is transported out of the cells to maintain a physiologically relevant GSH/GSSG redox balance. The activity of glucose-6-phosphate dehydrogenase (G6PD), a key enzyme in the supplementation of cells with NADPH, was also monitored.

AIMS

The aim of the study presented here:

- To investigate the effects of Cr(III) on the plasma membrane, the eukaryotic yeast cell seems a reasonable model.
- To investigate the biophysics and biochemists background of Cr(VI) sensitive phenotype with the stable Cr(VI) sensitivity of this mutant was caused by not a single gene mutation.
- To investigate the biophysics and biochemists background of Cr(VI) tolerant phenotype with the stable Cr(VI) tolerance of this mutant was caused by a single gene mutation.

METHODS

- The ergosterol-deficient *Candida albicans* mutant erg-2 (ATCC 44831) originated from the adenine-requiring ergosterol-producing wild-type strain 33 erg^+ (ATCC 44829). The Cr(VI)-sensitive mutant *chr-51S* was obtained from the auxotrophic strain *6 chr*+ (strain No. 6, *lys1-131 h*+), designated earlier as CS-6.51 and CW-6, respectively.
- Cell numbers were determined with a hemocytometer and OD values.
- The inhibitory effect of $CrCl_3$ on growth at different concentrations was measured in liquid MM cultures, cell numbers were determined with a hemocytometer.
- EPR spectra were recorded with an ESP 300E spectrometer (Bruker, Germany) equipped with an ER 412VT temperature regulator. The EPR spectra from the fatty acid spin label 5-SASL incorporated into the membranes were taken in the temperature range 0-40°C on both control and chromium-treated samples.
- Survival rates of cells exposed to the oxidative stressors H_2O_2, menadione (MD), *tert*-butyl hydroperoxide (*tert*-BOOH), and Cd_2^+ were estimated by a colony-counting assay.
- The specific intracellular activities of glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase G6PD, catalase, superoxide dismutases (Cu/Zn-SOD and Mn-SOD), and the specific intracellular concentrations of glutathione (GSH) and glutathione disulphide (GSSG), were determined by using well-established colorimetric assays.
- To estimate the intracellular peroxide and superoxide levels, the indicators dihydrorhodamine 123 (DHR 123) and dihydroethidium were used, the formation of rhodamine and ethidium (ET) was quantified spectrofluorimetrically and by flow cytometry, respectively. The *in vivo* generation and reduction of Cr(V) in *S. pombe* was followed by electron paramagnetic resonance (EPR) spectroscopy. The *in vivo* and *in vitro* formation of $^{\bullet}OH$ was measured by using the spin trap 0.1 M N-*tert*-butyl-*α*-phenyl nitrone (PBN).
- Data represent the mean of four independent experiments.

RESULTS

Interaction of Cr(III) with plasma membrane

- To investigate the interaction of yeast plasma membrane with Cr(III) ions, an ergosterol-producing 33 erg^+ strain and its ergosterol-deficient erg-2 mutant of *C. albicans* were used. The absence of ergosterol in the erg-2 mutant resulted in an increased accumulation of Δ8 sterols, a decreased fatty acid chain length, a lower proportion of unsaturated fatty acids, and an increased activity of membrane-bound chitin synthase activity in comparison with the 33 erg^+ strain. These differences in plasma membrane lipid composition of 33 erg^+ and erg-2 were reflected both in the phase transition of the membrane lipid extracts and in the phase transition measured on living cells. Ergosterol- producing 33 erg^+ exhibited higher membrane fluidity in comparison with mutant erg-2.
- The inhibition of growth caused by different $CrCl_3$ concentrations in shaken liquid media. A given $CrCl_3$ concentration had a significantly larger inhibition on the ergosterol-defficient mutant erg-2 than on its parental 33 erg^+ strain. Multiplication of both strains was blocked by 100 mM $CrCl_3$, but the cells remained viable at this concentration for 2 h. The viability of the cell population slowly decreased to zero by 160 h after treatment.
- Cr(III) treatment appeared to cause a loss of the barrier function of the plasma membrane, resulting in the leakage of low-molecular-mass substances from the cells. Treatment for 10 h caused a loss of metabolites absorbing at 260 nm; this loss was 40% for 33 erg^+ and 60% for erg-2.
- Cr(III) treatment caused an increased membrane fluidity in both strains, but this was more pronounced for the erg-2 mutant. The effect of the pH on the Cr(III)-treated membrane was studied by using the erg-2 mutant. Increase of the pH of the $CrCl_3$ solution from 2.15 to 4.30 decreased the fluidifying effect of Cr(III) on the membrane, but the tendency of the temperature changes was not modified.

Characterization of *chr1-51S* Cr(VI)-sensitive mutant

- The time-course of the reduction of Cr(V), followed by EPR spectroscopy, indicated that the *chr-51S* cells contained 2.5 times more Cr(V) than did those of its parental strain at the beginning of the EPR measurement, and underwent a much faster reduction of Cr(VI).

- The specific intracellular GSH concentration in the Cr(VI)-sensitive *chr-51S* mutant was about half that observed in the *6 chr*$^+$ parental strain.
- Cells pretreated with a sublethal dose of $K_2Cr_2O_7$ did not show any adaptive response either. In fact, this pretreatment increased the Cr(VI) sensitivity of the cells. On the other hand, adaptation to oxidative stress generated by 0.2 mM H_2O_2 resulted in significantly increased survival rates in the presence of $K_2Cr_2O_7$ concentrations.
- As regards the ROS metabolisms of the strains, the superoxide levels were about 1.6-times higher in the Cr(VI)-sensitive *chr-51S* mutant, which also oxidized the DHR 123 indicator stain much faster.
- As far as the GSH-dependent and GSH-independent elements of the antioxidative defence system are concerned, significantly higher GR and GST activities were recorded in the Cr(VI)-sensitive mutant than in its parental strain. It is noteworthy that the specific activity of the major NADPH supplier G6PD was also elevated in *chr-51S* cells. In *in vitro* PBN spin trapping EPR experiments, both *S. cerevisiae* and *S. pombe* purified GRs were shown to reduce Cr(VI) to Cr(V) effectively. *In vitro* Cr(VI)/Cr(V) redox cycling giving rise to considerable quantities of $^{\bullet}OH$ was also demonstrated in the presence of H_2O_2.
- The EPR results indicated the one-electron reduction of Cr(VI) to Cr(V) by GR isolated either *S. cerevisiae* or *S. pombe* and reoxidation of Cr(V) to Cr(VI) in the present of H_2O_2 via HABER-WEISS reaction.
- The same specific Cu/Zn-SOD, Mn-SOD, catalase and GPx activities were found in both parental and Cr(VI)-sensitive strains. Among the antioxidative enzymes, catalase was inducible by peroxides in both *6 chr*$^+$ and *chr-51S* cells, although the inducibility was much lower in the Cr(VI)-sensitive strain. Interestingly, the specific GPx activities did not respond to exposure to either H_2O_2 or *tert*-BOOH. Furthermore, the specific GR activities were not influenced by the addition of 0.5 mM diamide, which resulted in a 2.3-fold increase in the intracellular GSSG levels.

Characterization of *chr1-66T* Cr(VI)-tolerant mutant

- The time-course of Cr(V) formation and reduction monitored by EPR spectroscopy indicated that both strains reduced Cr(VI) to Cr(III) through Cr(V), but the elimination of Cr(V) in the Cr(VI)-tolerant *chr1-66T* mutant was much slower than in *6chr*$^{+}$ cells. The decreased reduction capacity of the Cr(VI)-tolerant mutant was also demonstrated *in vitro*. In this case, disrupted cells of both strains were exposed to 2.0 mM $K_2Cr_2O_7$ for 5 min; again, a significantly lower Cr(V) concentration was detected in the *chr1-66T* mutant than in the *6chr*+ strain. These experimental data supported the idea that there was a causal connection between the decreased reduction capacity of *chr1-66T* cells and their lower bioaccumulation of Cr(VI). These findings are in good accord with previous observations when the Cr(VI)-sensitive phenotype *S. pombe chr-51S* mutant was found to display an increased bioaccumulation of Cr(VI) and an accelerated reduction of Cr(V).
- The mutant *chr1-66T* was hypersensitive to the oxidative stress generated by several stressors, including H_2O_2, menadione, *tert*-BOOH and Cd_2^{+}.
- In fact, the intracellular GSH concentration found in the *chr1-66T* mutant was about half of that in the parental strain.
- Although the mutant strain possessed higher specific G6PD activity (G6PD is one of the key enzymes of the major NADPH-generating pentose phosphate pathway), the specific GR activity (which reduces GSSG at the expense of NADPH, a crucially important antioxidative enzyme) was highly reduced in *chr1-66T* cells. Since GR also reduces Cr(VI) directly to Cr(V), with the concomitant formation of superoxide, and its overproduction results in a Cr(VI)-sensitive phenotype, decreased GR activities are expected to lead to the manifestation of a Cr(VI)-tolerant and oxidative stress-sensitive phenotype, as observed with the *chr1-66T* strain.
- Besides the decreased specific GR activity, the increased oxidative stress-sensitivity of the Cr(VI)-tolerant mutant could be a consequence of the decreased mitochondrial Mn-SOD activity giving rise to high intracellular superoxide levels. *S. pombe* contains two superoxide dismutases, one in the cytosol (Cu/Zn-SOD) and the other in mitochondria (Mn-SOD). Mn-SOD encoded by *sod2*$^{+}$ gene is a particularly important element of the antioxidative defence system, preventing the generation of superoxide in metabolically active mitochondria. The decreased disproportionation of $O_2^{\bullet -}$ to

H_2O_2 and O_2 by Mn-SOD most probably contributed to the increased Cr(VI) tolerance of *chr1-66T* too.

SUMMARY; PRESENTATION OF NOVEL FINDINGS

- The effects of Cr(III) ions on the cell membranes were found to depend on concentration and time, and the results were influenced by the membrane composition. The consequences of Cr(III)-induced membrane fluidity might be due to the damage to the barrier function disturbing the homeostasis of the cells and leading to cell death. It seems that the Cr(III) cation has a preferred localization in the negatively charged regions of the membrane, and tends to create bridges between the phospholipid molecules and the side-chains of the negatively charged amino acid residues. These interactions reduce the stability of the head group regions of the membrane, decrease the rotational energy barriers for spin probes, and possibly affect the bound layer of phospholipids around the membrane proteins. No marked dipolar broadening was observed in the presence of Cr(III), indicating that the magnetic interaction between the spin probes and Cr(III) ions can be neglected.
- The time-course of the reduction of Cr(V), followed by EPR spectroscopy, indicated that the *chr-51S* cells contained 2.5 times more Cr(V) than did those of its parental strain at the beginning of the EPR measurement (data not presented), and underwent a much faster reduction of Cr(VI).
- Cells pretreated with a sublethal dose of $K_2Cr_2O_7$ did not show any adaptive response.
- The Cr(VI)-sensitive phenotype of *S. pombe chr-51S* cells was a consequence of changes in the metabolic network of the yeast, including (i) a significantly increased GR/G6PD activity that accelerated the reduction Cr(VI) → Cr(V) and hence facilitated the intracellular accumulation of Cr, (ii) a reduced intracellular GSH concentration that hindered the effective elimination of •OH from the cells, and (iii) an underregulated catalase activity that would have been crucial to block the Fenton-type formation of •OH.
- As far as the GSH-dependent and GSH-independent elements of the antioxidative defence system are concerned, significantly higher GR and GST activities were recorded in the Cr(VI)-sensitive mutant than in its parental strain. The catalase of the mutant was less inducible relative to that of the parental strain, which further

weakened the oxidative resistance of the mutant. Catalase is basically important to decompose intracellular H_2O_2 and therefore to hinder Fenton-type processes resulting in $^{\bullet}OH$. However, *in vivo* experiments with disrupted cells proved that in spite of lower GSH content of the *chr-51S* mutant its increased GR and G6PD activities resulted significantly higher capacity to generate $^{\bullet}OH$ than its parental strain in the presence of chromate and elevated intracellular peroxide concentration.

- The Cr(VI) tolerant mutant strain possessed higher specific G6PD activity, the specific GR activity was highly reduced in *chr1-66T* cells. Since GR also reduces Cr(VI) directly to Cr(V), with the concomitant formation of superoxide, and its overproduction results in a Cr(VI)-sensitive phenotype, decreased GR activities are expected to lead to the manifestation of a Cr(VI)-tolerant and oxidative stress-sensitive phenotype, as observed with the *chr1-66T* strain. Although the addition of NADPH to Cr(VI)-treated disrupted cells of *chr1-66T* resulted in a major increase in the generation of Cr(V).
- In conclusion, the majority of the data presented here can be explained in terms of a mutation affecting the one-copy GR gene itself or its transcriptional regulation, including the observed Cr(VI) tolerance and oxidative stress sensitivity. The decreased Mn-SOD activity enhanced both effects. Experiments aimed at the construction and characterization of GR overproducing transformants of *chr1-66T*, and the sequence analysis of the *chr1-66T pgr+1* gene, are the next steps in this project.

LIST OF PUBLICATIONS

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