Distribution of $^{64}$Cu in *Saccharomyces cerevisiae*: cellular locale and metabolism

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The metabolism of copper in the yeast *Saccharomyces cerevisiae* has been studied with respect to the distribution and stability to exchange of newly arrived $^{64}$Cu. Cells pre-incubated with 10 μM-$^{64}$Cu$^{2+}$ accumulated $^{64}$Cu into two pools distinguishable by cellular locale and lability to exchange with extracellular cold copper. One pool was non-exchangeable and was localized to protoplasts. Size-exclusion chromatography of a soluble cell (protoplast) extract showed that this $^{64}$Cu was associated with up to four species. Two were identified as copper metallothionein and Cu,Zn superoxide dismutase based on comparisons of chromatograms derived from strains in which the genes for these two proteins had been deleted. A third species was identified as copper-glutathione based on chromatographic and biochemical assays. A second pool was exchangeable and was localized to the cell wall. In contrast to its rapid copper-stimulated exchange ($t_{1/2}$ ≈ 1 min), this pool exhibited only slow efflux (10% $^{64}$Cu loss per 60 min). Zn$^{2+}$ did not stimulate the loss of $^{64}$Cu from this pool indicating that it was selective for copper. This pool was released into the supernatant upon protoplast formation and was found in the cell wall debris obtained when cells were mechanically disrupted. This $^{64}$Cu eluted in the void volume (peak P,) of the column used to size-fractionate copper-binding species. The metal in P, was exchangeable *in vivo* and *in vitro*. However, the corresponding chromatographic fraction obtained from copper-naive cells when labelled *in vitro* could bind less than 20% of the $^{64}$Cu bound to it *in vivo* indicating that the deposition of copper in this pool was primarily cell-dependent. In fact, this deposition was shown to be dependent on the cellular reduction of medium sulphate or sulphite to the level of sulphide, or on the addition of sulphide to the $^{64}$Cu uptake buffer. $^{64}$Cu in the non-exchangeable protoplast pool was not mobilized by cellular sulphide generation, indicating that cellular sulphide generation did not causally lead to the partitioning of $^{64}$Cu to the cell wall pool. The data indicate that the appearance of copper sulphide(s) on the cell wall in *S. cerevisiae* is gratuitous and does not represent a sulphide-based mechanism of copper resistance in this yeast.

Introduction

The accumulation and detoxification by yeasts and fungi of metal ions such as Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, and Ag$^+$ have been characterized in some detail. Interest in these processes stems from the potential use of such organisms in biological waste treatment (Gadd, 1988; Macaskie & Dean, 1989) and from the desire to delineate the metal-dependent, self-regulatory aspects of the metal-handling pathways involved (Silver *et al.*, 1989; Winge & Mehra, 1991). Thus, yeasts and fungi exhibit an acquired resistance to these metal ions which can involve one or more cellular elements. For example, the expression of cysteine-rich protein(s), commonly designated metallothionein, is a relatively ubiquitous cell response to elevated environmental levels of these first- and second-row metals (Karin, 1985; Hamer, 1986; Petering & Fowler, 1986). In some lower eukaryotes another presumed detoxification/resistance mechanism involves the generation of H$_2$S which can either intra- or extracellularly trap the metal ion as an insoluble sulphide (Ashida *et al.*, 1963; Kikuchi, 1965; Pan-Hou & Imura, 1981). Metal sulphides have been observed microscopically in such strains following exposure to elevated metal concentrations (Ashida *et al.*, 1963). Another type of liganding species is represented by the cysteine-rich $\gamma$-glutamyl peptides synthesized by a number of organisms including the yeast *Schizosaccharomyces pombe* (Reese *et al.*, 1988). In the yeast *Candida glabrata* these peptides,
as well as glutathione, serve as coatings for a CdS crystallite (Dameron et al., 1989).

Although much is established about the ways in which a cell can sequester a metal ion such as Cu\(^{2+}\), understanding of the pathway(s) by which the metal is partitioned to the various cellular sites of utilization and deposition is limited. Some information about the overall distribution of metal has been published, however (White & Gadd, 1986). As noted, \( \text{H}_2\text{S} \) generation has been proposed to causally lead to the deposition of copper in or on the cell wall, thus providing an important copper resistance mechanism in some yeasts (Kikuchi, 1965; Ashida, 1965). In the yeast *Debaryomyces hansenii*, copper binding to the cell wall has been ascribed to Cu(II) reductase activity in this locale; the Cu(I) bound there is suggested to be available for subsequent intracellular utilization (Wakatsuki et al., 1988). These suggestions have not been rigorously evaluated by the appropriate distribution and kinetic analyses, however.

In contrast, a detailed evaluation of copper uptake (transport) and accumulation (retention) by wild type and copper thiorein-deleted strains of *Saccharomyces cerevisiae* provided some evidence for a copper metabolic pathway in this yeast (Lin & Kosman, 1990). This pathway involved the partitioning of newly cell-associated \(^{64}\text{Cu}\) into a kinetically stable, non-exchangeable, probably intracellular pool and a \(^{64}\text{Cu}\) fraction in exchange with extracellular Cu\(^{2+}\) which appeared to be external to the plasma membrane. The data showed that all \(^{64}\text{Cu}\) accumulated by cells grown in the virtual absence of copper (copper-naive cells) represented an increase in total copper rather than isotopic exchange. This accumulation was kinetically saturable and energy-dependent. De Rome & Gadd (1987) also have demonstrated that copper uptake in *S. cerevisiae* is a facilitated, perhaps active, transport process. Cells previously exposed to copper, however, exhibited an isotopic exchange as well as net copper uptake; this apparent isotopic exchange was energy-independent and not inhibited by cycloheximide (Lin & Kosman, 1990). In contrast, cycloheximide inhibited the net uptake of copper by copper-naive cells and blocked the appearance of this exchangeable pool, indicating as well that the development of this latter pool required prior exposure to copper. This report presents in some detail those distribution studies needed to more fully characterize the cellular trafficking of the \(^{64}\text{Cu}\)\(^{2+}\) which becomes newly cell-associated in the yeast *S. cerevisiae*.

**Methods**

**Chemicals.** Media components were from Difco. Zymolyase 100T was purchased from ICN. Purified yeast Cu,Zn superoxide dismutase was from Pharmacia; yeast copper thiorein was purified by the method of Winge et al. (1985). The reagents for preparing the completely defined synthetic medium used for growth of copper-free cultures and the MES for buffering the uptake medium were AnalaR or AristaR grade chemicals (BDH) purchased from Gallard-Schlesinger. Media supplements and reagents for the sulphite reductase and glutathione assays were purchased from Sigma. The copper standard for atomic absorption spectrophotometry was purchased from Alfa. \(^{64}\text{Cu}\)\(\text{NO}_3\)\(_2\) was supplied by the Buffalo Materials Research Center, SUNY at Buffalo, NY, USA. Solutions were prepared in doubly deionized, doubly (glass) distilled water.

**Medium for cell growth.** Yeast stocks were maintained on YPD medium [yeast extract, 1 % (w/v); bacto-peptone, 2 % (w/v); dextrose, 2 % (w/v)]. Cultures for experiments were grown in a synthetic, complete medium prepared without sulphate (Difco Manual, 1984, 10th edn, pp. 1135–1141; Greco et al., 1990; Lin & Kosman, 1990) but with the addition of 0.1 M-Tris/succinate which maintained the pH of the culture at 6.0 throughout growth. This medium contained approximately 15 nm-copper as determined by flameless atomic absorption spectrophotometry (AAS) and is referred to as copper-free medium. This level of copper is at the detection limit of AAS. For reference, normal minimal medium (based on yeast nitrogen base) contained 150–200 nm-copper, whereas YPD medium contained 275–325 nm. Sulphate was added to this medium as Na\(_2\)SO\(_4\) and Cu\(^{2+}\) as CuCl\(_2\), as desired. The Cu\(^{2+}\) added to the synthetic medium was probably present as Cu(I)\(^{2+}\) bound to the amino acids in the medium, particularly histidine. The carbon source in this medium was dextrose (2%, w/v).

**Yeast strains and genotypes.** The wild type strain used for most experiments was AS2-2A (MATa trp1-2 MAL4). It exhibits the copper resistance phenotype characteristic of a strain which is also CUP\(^R\) (Greco et al., 1990). The CUP1- and SOD1-deleted strains used were: 51-2-2 (Hamer et al., 1985: MATa trp1-1 gal1 met13 can1 ura3-50 ade8 his3 URA3: URA3) and EGI (Gralla & Valentine, 1991: MATa leu2-3,112 his3A1 trp1-289 ura3-52 SOD1::URA3), constructed from strain DBY746. Two methionine auxotrophs were used, both of which were obtained from the Yeast Genetics Stock Center, University of California, Berkeley, CA, USA. These were: S30 (MATa met4 leu2) and STX-4-4D (MATa met14 his3 ade4 his6 leu2 gal2).

**Cell growth.** Cell growth was monitored by measuring OD\(_{600}\). Liquid stocks were prepared using washed single colonies taken from YPD plates as inocula. These stocks were kept in the copper-free synthetic medium. Growth of experimental cultures was initiated at OD\(_{600} = 0.1 \) or less; the cultures were allowed to grow to OD\(_{600} = 0.8–1.2\) (exponential phase, 1-2 x 10\(^6\) cells ml\(^{-1}\)).

\(^{64}\text{Cu}\)\(^{2+}\)-labelling protocol. Cells for labelling experiments were washed with uptake buffer (5 x culture volume) and resuspended in the uptake buffer at the OD\(_{600}\) indicated in the figure legends which was usually 1.0. This buffer contained 0.2 % MES (pH 6.0) and glucose (2%, w/v) which together maintained the solution at 330 mOsm (determined using a Precision Systems freezing point depression micro-osmometer), the same osmolarity as the growth medium. This buffer contained no detectable copper (<15 nm). Following a 15 min equilibration in this buffer at 30 °C, the bis-histidine complex of Cu\(^{2+}\), \(^{64}\text{Cu}\)His\(_2\), was added to initiate uptake. This complex was prepared by incubating 10 part Cu(NO\(_3\))\(_2\) with 2.1 parts histidine in uptake buffer. Cell-associated \(^{64}\text{Cu}\) was determined by first quenching samples (1 ml) in 5 ml of an ice-cold solution of 10 mM-EDTA in 0.1 M-Tris/succinate, pH 6.0. These samples were filtered by suction through nitrocellulose (0.45 μm, Millipore) and washed with a total of 20 ml quench solution. The filters were allowed to air dry and then were counted in an LKB CompuGamma gamma counter. Counts were corrected for background, efficiency and decay to yield ng copper. For experiments in which cells were preincubated with cold Cu\(^{2+}\), the addition was to cultures in growth medium. \(^{64}\text{Cu}\) was then determined as described.
following the washing and resuspending of the cells in uptake buffer. Analysis of net uptake using atomic absorption spectrophotometry was carried out in much the same way except that the quenched cell sample was washed at 4 °C by centrifugation using the quench buffer. The resulting cell pellet was resuspended in water and analysed by flameless atomic absorption spectrophotometry either directly or following an acid digestion in HClO₄.

Preparation of cell extracts and protoplasts. Soluble cell extracts were prepared by vigorous vortexing of about 10⁷ cells with 50 µl buffer (10 mM-sodium phosphate, pH 7.8) and an equal volume of glass beads (0.3–0.4 mm, Thomas Scientific). Following centrifugation, the supernatant was removed and the pellet re-extracted with an additional 50 µl of buffer, the second supernatant was combined with the first and stored at −70 °C. Larger volumes of cells were extracted similarly, increasing the volumes noted proportionally. Generation and isolation of protoplasts was carried out as described by Kuo & Yamamoto (1975).

Chromatographic analysis of soluble copper-binding species. Cell extracts were prepared from cultures grown at both [Cu⁺²]₀ = 10 µM and approximately 200 nM (normal synthetic complete medium). These extracts were size-fractionated on an HR 10/30 Superose 12 column (Pharmacia) employing an LKB/Pharmacia FPLC. The eluting buffer was 0.15 M-NH₄HCO₃, pH 7.8; the flow rate was 0.4 ml min⁻¹. Typically, 0.05–1.0 mg protein in 170 µl was applied to the column; 200 µl fractions were collected after 6 ml flow-through. The copper-content of the fractions was determined as ⁶⁴Cu by gamma counting, by flameless atomic absorption spectrophotometry, or by both methods. Replicate samples were analysed in all cases (n ≥ 3). The resulting analyte level in each fraction was accurate to within ±4%. We found that Superose 12 binds free or loosely bound Cu⁺² avidly. As a result, except when present at ≥ 100 µM, little ⁶⁴Cu was detected as free copper (approximately fraction 80). To limit potential cross-contamination of chromatographs, the column was washed with 0.1 M-HCl between runs. ⁶⁴Cu bound to proteins, histidine or glutathione was stable to chromatography, however. Consequently, recovery of ⁶⁴Cu in cell extracts and in vitro-labelled complexes following chromatographic separation was routinely 96–104%.

Analytical methods. Atomic absorption analyses were performed on a Perkin Elmer Model 1100B spectrophotometer equipped with a model 700 graphite furnace and an AS-70 auto-sampler. The limit of detection for copper was approximately 1 p.p.b. (≈ 50 pg per 50 µl, the limiting sample volume). As noted above, this was equivalent to 15 nm-Cu. Total cell protein was determined on all cell samples analysed for copper accumulation (Stewart, 1975), whereas protein in soluble extracts was determined by BCA (bicinchoninic acid) assay (Pierce) using bovine serum albumin as standard (Smith et al., 1985). A crude sulphite reductase fraction was isolated and assayed as described by Yoshimoto & Sato (1968). A glutathione reductase assay (Anderson, 1985) was used to determine total glutathione levels in cell extracts and chromatographic fractions. Cell viability following all treatments was determined by standard dilution and plating techniques on YPD. Dilutions were adjusted to yield 50–100 colonies per plate. In no case was cell viability at the end of an experiment less than 90% of either t₀ or control cells. Statistical analyses were performed using the INSTANT program from GraphPAD (San Diego, CA, USA).

Results

⁶⁴Cu efflux and exchange from S. cerevisiae pre-loaded with ⁶⁴Cu⁺²

We have shown previously that S. cerevisiae pre-incubated with 10 µM-Cu⁺² and then challenged with ⁶⁴Cu⁺² exhibits a fast (t₁ < 1 min), energy-independent but specific accumulation of ⁶⁴Cu⁺² which could be ascribed to isotopic exchange rather than net uptake (Lin & Kosman, 1990). This phenomenon is illustrated also by the data shown in Fig. 1(a). Wild type strain AS2-2A grown in copper-free medium ([Cu] ≤ 15 nM) was incubated with 10 µM-⁶⁴Cu-His₉ for 30 min. The culture was washed and resuspended in the MES-glucose uptake buffer. In the absence of added cold copper (●) or in the presence of 50 µM-Zn⁺² (○) less than 25% of the label was lost in 120 min at 30 °C. In contrast, 75 ± 5% of the label was chased from the cells by 10 µM-Cu⁺² within 2 min (□) indicating that the apparent isotopic exchange is specific for Cu⁺². Cells incubated with azide (350 µM) prior to the addition of cold Cu⁺² quantitatively exhibited the same rapid loss of ⁶⁴Cu. This is in contrast to the 50%
inhibition of net $^{64}\text{Cu}$ uptake by azide in glucose-grown (fermenting) cells (Lin & Kosman, 1990), indicating that the copper-stimulated loss of $^{64}\text{Cu}$ ($\bigcirc$) is energy-independent. When the cold copper chase was conducted at 4 °C the $t_1$ for exchange was increased to approximately 20 min, consistent with the 26 °C decrease in temperature ($\blacksquare$). The temperature dependence is consistent with the apparent isotope exchange occurring external to the plasma membrane since, as shown previously (Lin & Kosman, 1990), net uptake by yeast cells is negligible at 4 °C.

To determine if the slow loss of cell-associated $^{64}\text{Cu}$ in the absence of added copper (Fig. 1 a, $\blacklozenge$) was related to the isotope pool in apparent fast exchange ($\square$), labelled cells were first incubated in copper-free buffer for 60 min prior to the 10 μM-cold copper chase (Fig. 1 b, $\blacklozenge$). These cells lost 22 ± 4% of the initial $^{64}\text{Cu}$ during this period ($\blacklozenge$, $t = 0$ in Fig. 1) but reached the same end point (28 ± 4% retained) after the chase as did cells challenged with the cold copper without a pre-efflux period (Fig. 1 b, $\bigcirc$). This result suggests that the copper slowly lost by cells (Fig. 1 a, $\blacklozenge$ and b, $\blacklozenge$, $t = 0$) and that which is rapidly exchangeable is from the same pool or from pools which are in rapid equilibrium with one another.

**Cellular locale of the exchangeable and non-exchangeable $^{64}\text{Cu}$-binding pools**

The possibility that the exchange pool of copper was periplasmic was assessed by disrupting $^{64}\text{Cu}$-labelled cells in two different ways both before and after a cold copper chase: mechanically, by grinding with glass beads, and by treatment of the cells with Zymolyase which releases the protoplasts from a partially digested cell wall ‘ghost’. In the latter treatment, the protoplasts and cell wall debris can be separated by differential centrifugation; the final supernatant contains soluble periplasmic components and any soluble species released from the cell wall as a result of its partial proteolysis. The pellet resulting from mechanical disruption contains both cell wall and membrane components, including those from intracellular organelles. This pellet can also contain intact cells; the fraction of intact cells remaining was estimated to be $\leq 5\%$ based on microscopic examination. These fractions prepared from labelled cells were challenged also with cold copper. The results of these various manipulations are summarized in Table 1.

In sample A, experiment 1, the cells were mechanically disrupted before the chase. The majority of the cell-associated $^{64}\text{Cu}$ (86%) was found in the insoluble (cell wall/membrane) fraction. Cold copper added in a secondary treatment of this fraction chased out 80% of the label retained initially (18 versus 86%). Sample B was treated with cold copper prior to mechanical disruption as a secondary treatment. In the 1 min chase, 68% of the label was lost (32% of the total $^{64}\text{Cu}$ retained). The secondary treatment showed that this label came entirely from the insoluble fraction (20 versus 86% of the original $^{64}\text{Cu}$ retained); the soluble fraction lost an insignificant amount of $^{64}\text{Cu}$ (12 versus 14% of the original $^{64}\text{Cu}$ retained). In experiment 2, sample A, Zymolyase treatment of labelled cells yielded protoplast, cell wall debris and supernatant fractions. The majority of cell-associated $^{64}\text{Cu}$ was found in the supernatant fraction. This fraction of $^{64}\text{Cu}$ represented the exchangeable pool since it was essentially all lost if the labelled cells were challenged with the chase prior to secondary treatment with Zymolyase (sample B). In contrast, of the 18% of the total $^{64}\text{Cu}$ in the protoplasts obtained from sample A, 80% was stable to exchange when the protoplasts themselves were exposed to a secondary treatment with 10 μM-Cu²⁺ (14 versus 18%). The cell wall debris from the Zymolyase treatment had relatively little associated $^{64}\text{Cu}$; it appeared exchangeable, however (samples A and B, 7 versus 1%).

Three other aspects of these data should be noted. First, comparison of sample A from experiments 1 and 2 indicates that little soluble $^{64}\text{Cu}$ was periplasmic since the soluble $^{64}\text{Cu}$ in sample A, experiment 1 (14%) can be accounted for by the $^{64}\text{Cu}$ found in the protoplasts obtained in sample A, experiment 2 (18%). Second, the insoluble fraction from mechanically disrupted cells (86% of the cell total, sample A, experiment 1) could contain copper associated with insoluble components other than the cell wall, e.g. (intra)cellular membranes. However, such copper does not appear to make a significant contribution to this fraction. This conclusion is based on the fact that following Zymolyase treatment, 7% of total cell-associated $^{64}\text{Cu}$ was found in the cell wall debris, whereas 75% was in the supernatant; only 18% remained with(in) the protoplast (sample A, experiment 2). The inference that the 75% of total $^{64}\text{Cu}$ in this supernatant came from the cell wall was confirmed since a secondary treatment with Zymolyase of the insoluble fraction from sample A, experiment 1 (primarily cell wall) released 97% of the associated $^{64}\text{Cu}$ (data not shown). Finally, the fact that the distribution profiles were quantitatively independent of the method of cell fractionation suggests that redistribution of $^{64}\text{Cu}$ was not significantly altering the results.

**Molecular characterization of the exchangeable and non-exchangeable $^{64}\text{Cu}$-binding pools**

The nature of the copper-binding species associated with these two copper-binding pools was evaluated qualitatively by chromatographic analysis of soluble fractions derived from whole cells (mechanically disrupted), of
Table 1. Cellular locale of exchangeable and non-exchangeable 64Cu-binding pools

Wild type strain AS2-2A was grown to early exponential phase in copper-free medium, washed and resuspended in the MES-glucose buffer. 64Cu-His2 (10 μM) was added and the cultures incubated for 30 min. Cells were then washed and either mechanically disrupted (experiment 1, sample A) or treated with Zymolyase (experiment 2, sample A) or were washed, and challenged with 10 μM-Cu2+ prior to cell fractionation (samples B). In two cases, labelled cell components were challenged with the cold copper chase (experiment 1, sample A insoluble fraction, and experiment 2, sample A protoplasts). The percentage total 64Cu retained was based on the initial, total cell-associated 64Cu. This value was typically 2000 ± 200 c.p.m. per 2 × 10^9 cells. The values are means (±SEM) derived from three separate experiments.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Percentage total 64Cu retained</th>
<th>Percentage total 64Cu retained</th>
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<tr>
<td>Sample A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical disruption yielded:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insoluble fraction</td>
<td>86±6</td>
<td>Secondary 10 μM-Cu2+ chase in the insoluble fraction</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>14±3</td>
<td></td>
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<tr>
<td>Sample B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μM-Cu2+ chase in whole cells</td>
<td>32±5</td>
<td>Secondary mechanical disruption yielded:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insoluble fraction</td>
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<tr>
<td></td>
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<td>Soluble fraction</td>
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<td>Sample B</td>
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<tr>
<td>10 μM-Cu2+ chase in whole cells</td>
<td>20±3</td>
<td>Secondary Zymolyase treatment yielded:</td>
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<td>Protoplasts</td>
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<td>Sample A</td>
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<td>Zymolyase treatment yielded:</td>
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<tr>
<td>Protoplasts</td>
<td>18±3</td>
<td>Secondary 10 μM-Cu2+ chase in the protoplasts</td>
</tr>
<tr>
<td>Cell wall debris</td>
<td>7±2</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>75±6</td>
<td></td>
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<tr>
<td>Sample B</td>
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<tr>
<td>10 μM-Cu2+ chase in whole cells</td>
<td>20±3</td>
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The behaviour of P1, P3, and P6 with respect to isotope exchange was also studied in two experiments. First, labelled wild type cells (DBY746, as in Fig. 2b, ○) were exposed to a 10 μM-Cu2+ chase. An extract was prepared from these cells and chromatographed (Fig. 2c, ○). Clearly, the 64Cu in these three copper-binding fractions was exchange-labile in vivo since less than 10% of 64Cu in these fractions was retained (cf. b and e, ○). These fractions were exchange-labile in vitro, also. This was shown in a second experiment in which 64Cu2+ (as 64Cu-His2) was added back to this extract from DBY746 prepared following the Cu2+ chase. This re-labelled extract yielded a chromatogram which was quantitatively similar to that obtained with the extract labelled in vivo (cf. Fig. 2c, ○ to b, ○). These results show that although the copper in the protoplast is not exchangeable with the extracellular space (see Table 1, experiment 2, sample A) the sites to which the metal is bound within the protoplast (cell) are exchange-labile. In this sense, the term 'non-exchangeable pool' is accurate only with respect to vectorial movement of copper out of the cell, that is, once copper passes into the cytoplasm, it does not have an efficient mechanism to get back out. Significantly, the

soluble lysates from protoplasts, and of the supernatant from Zymolyase-cell incubation mixtures. Soluble extracts from whole cells labelled with 10 μM-64Cu were obtained from both wild type strains and strains which carried a complete deletion in the gene for one of the two known, soluble copper-binding proteins in yeast, copper metallothionein (CUP1, see Hamer et al., 1985) and Cu,Zn superoxide dismutase (SOD1, see Gralla & Valentine, 1991). These extracts were analysed by size exclusion FPLC on Superose 12. The resulting chromatograms are shown in Fig. 2. Comparison of the chromatogram associated with wild type strain 19.3C (a, ○) with the one associated with the CUP1-deleted strain, 51.2-2 (a, ●) showed that the 64Cu-binding peak eluting at fraction 46 (P25) was due to copper metallothionein. A similar comparison (b) involving wild type strain DBY746 (○) and the SOD1-deleted strain, EG1, derived from DBY746 (●) showed that P32 was due to Cu,Zn superoxide dismutase. Soluble lysates derived from protoplasts yielded chromatograms which were essentially identical to those shown in Fig. 2 with the exception that the fraction P, was absent in all cases (data not shown).
isotope exchange seen in cell extracts is not observed with the purified proteins. $^{64}$Cu incubated for 2 h with yeast Cu,Zn superoxide dismutase or copper thionein in buffer was not incorporated into either protein as detected by subsequent chromatographic analysis (data not shown).

Two features of results like those in Fig. 2 should be noted. First, the relative amount of $^{64}$Cu associated with the various copper-binding species resolved varied significantly between yeast strains. Second, but related to the first point, three other copper-associated peaks were observed, but in highly variable relative amounts, in these chromatograms and ones derived from other strains, namely, a fraction which eluted in the void volume ($P_v$), one which eluted at fraction 22 ($P_{22}$, not seen in the strains represented here) and $P_{56}$.

The apparent molecular mass of $P_{56}$ was 4.0 ± 0.2 kDa; this fraction eluted prior to ‘free’ ionic copper ($P_{in}$, see Methods) and $^{64}$Cu·His$_2$ (P$_{61}$, molecular mass 1.7 ± 0.2 kDa) suggesting that this species was a complex of copper and glutathione (Germann & Lerch, 1987; Freedman & Peisach, 1989; Freedman et al., 1989). This possibility was strongly suggested by two results. First, assay for glutathione across the $P_{56}$ peak revealed that $^{64}$Cu and GSH/GSSG co-eluted. The ratio of total glutathione to total copper in this peak was 8:1. In contrast, no glutathione was detected in any of the other peaks of $^{64}$Cu. Second, Cu·GSH (prepared from a 10:1 molar ratio of the reduced ligand and metal, cf. Cirioio et al., 1990) exhibited the same elution volume as did $P_{56}$. Note that these results do not exclude the possibility that $P_{56}$ is heterogeneous, however.

Chromatographic analysis of the supernatant fraction from experiment 2, sample A (periplasmic compounds released by Zymolyase treatment) indicated that the $P_v$ fraction was associated with the exchangeable $^{64}$Cu-binding pool. The $^{64}$Cu appearing in this fraction was chased out when either the cells (see experiment 2, sample B) or the Zymolyase supernatant was challenged with unlabelled copper, demonstrating that this fraction represented an exchangeable copper-binding pool (data not shown). Similarly, if the $^{64}$Cu in the $P_v$ fraction as in Fig. 2 (a, O) was allowed to decay and the pooled fractions then re-labelled with 10 μM-$^{64}$Cu$^{2+}$ and re-chromatographed, a quantitatively identical peak of $^{64}$Cu binding was obtained, again demonstrating exchange-lability (data not shown). Atomic absorption analysis of these fractions showed that the mass of copper was not increased by this in vitro incubation with Cu$^{2+}$ which would have occurred if the added $^{64}$Cu$^{2+}$ were binding to unoccupied sites. In contrast, if a Zymolyase supernatant prepared from copper-naive cells was incubated with up to 100 μM-$^{64}$Cu$^{2+}$ and then chromatographed, the fraction corresponding to $P_v$ retained less than 20% of the $^{64}$Cu that was associated with this fraction when derived from copper-treated cells labelled in vivo (data not shown). This result suggests that $^{64}$Cu binding to this fraction appears to be a cell-dependent process.
Genetic and biochemical characterization of the exchangeable, cell wall-associated $^{64}$Cu-binding pool

Periplasmic metal accumulation in fungi has been associated with cellular production of H$_2$S (Kikuchi, 1965; Ashida, 1965). The hypothesis that the exchangeable, apparently cell wall $^{64}$Cu-binding pool demonstrated here was associated with sulphide generation was tested by both metabolic and genetic approaches. Sulphide is an intermediate (by-product) in the reductive assimilation of sulphate as illustrated in Fig. 3. The genes for the enzymes in this pathway have been identified and mutants in all of these genes have been isolated. The transcription of several of these genes is repressed by S-adenosylmethionine (SAM), one of the end-products of the assimilation pathway (Jones & Fink, 1982). Thus, sulphide production can be suppressed by Met (1 mM) or SAM (0.2 mM) when either is added to the growth medium at the concentrations indicated (Thomas et al., 1989); is absent in cells grown in sulphate-free media; and is limited or absent in mutants which lack an enzyme activity upstream from sulphide in the pathway. We have exploited these features to show that sulphide generation is required for the appearance of the exchangeable $^{64}$Cu-binding pool.

The wild type strain, AS2-2A, which is a Met prototroph, was grown in a completely synthetic medium with addition of sulphate and/or Met as noted in Table 2. Total $^{64}$Cu accumulation by, and the cellular distribution of this $^{64}$Cu in these various cultures were determined in MES-glucose buffer which contained the additions as noted. The data shown in Table 2 indicate first that, irrespective of additions to the growth medium, total $^{64}$Cu accumulation and accumulation in the exchangeable pool was correlated to the amount of sulphate in the uptake buffer. In contrast, $^{64}$Cu accumulation in the non-exchangeable pool was relatively insensitive to the presence of sulphate.

Fig. 3. Sulphide formation by S. cerevisiae and its regulation. The steps in the reductive assimilation of sulphate up to the generation of sulphide and the corresponding enzymes and genes (as mutant alleles) are illustrated. The central role of the end-product SAM in the repression of expression of three of the four structural genes involved in the generation of sulphide is indicated.

With respect to the effects of additions to the growth medium, the data also show that even in the presence of sulphate, Met suppressed total $^{64}$Cu accumulation; this decrease was primarily accounted for by a sixfold suppression of $^{64}$Cu accumulation in the exchangeable pool. There was a further 14-fold decrease in this pool when sulphate was removed from the medium. The decrease seen in the presence of sulphate plus Met (six-fold) can be compared to the decrease in sulphite reductase activity due to repression of MET5,10 by SAM (Fig. 3). Addition of Met to the medium caused a decrease in this activity from 11.5±0.6 to 1.5±0.3 μmol min$^{-1}$ (mg protein)$^{-1}$ ($n=3$) in a crude sulphite reductase preparation (see Methods). Similar results were obtained when SAM rather than Met was added to the growth medium (data not shown) indicating that these effects were not due to Met specifically. Other forms of sulphur were added to the sulphate-free wild type cells in the uptake buffer as well. Sulphite increased the exchangeable pool 20-fold, whilst sulphide increased it by 150-fold. Similar to the uptake buffer, the growth medium composition had no systematic effect on the amount of $^{64}$Cu in the non-exchangeable pool (Table 2).

Strains S30 and STX4-4D carry mutations in the MET3 (ATP sulphurylase) and MET14 (APS kinase) genes, respectively (Fig. 3). Neither can convert sulphate to sulphide, thus both are Met auxotrophs. The distribution of cell-associated $^{64}$Cu in these strains was determined as above. These data are given in Table 2 also and show that the exchangeable pool of $^{64}$Cu was only 5% (S30) to 15% (STX4-4D) of that in the wild type strain (cf. AS2-2A grown in the presence of Met+ sulphate). On the other hand, with sulphite added to the uptake medium, this pool in the two mutant strains was recovered (Table 2, cf. sulphate versus sulphite). Also, chromatographic analysis of the supernatant fractions obtained following Zymolyase treatment of $^{64}$Cu-labelled Met$^-$ strain S30, or wild type strain AS2-2A labelled in
Table 2. Active sulphide generation and accumulation and distribution of $^{64}$Cu

Strains were grown to early exponential phase in a synthetic medium which was either sulphate-depleted or contained sulphate (10 mM) and/or Met (1 mM) as indicated. Cells were washed and resuspended in the MES-glucose uptake buffer in the presence of additions as indicated. The cultures were incubated with $^{64}$Cu-His$_2$ (10 $\mu$M) for 30 min, washed and counted to determine total accumulation of $^{64}$Cu. They were then resuspended in the uptake buffer and challenged with 10 $\mu$Mcold Cu$^{2+}$ for 20 min. The fraction of non-exchangeable $^{64}$Cu was determined by counting the cells, whilst the fraction of exchangeable $^{64}$Cu was calculated by difference. The values are means (SD±4.9%, n = 3) from one experiment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additions to growth medium</th>
<th>Additions to uptake buffer</th>
<th>Total $^{64}$Cu (nmol)</th>
<th>Exchangeable $^{64}$Cu (nmol)</th>
<th>Non-exchangeable $^{64}$Cu (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS2-2A (Met')</td>
<td>$\text{SO}_4^{2-}$ (Met)</td>
<td>None</td>
<td>1.33</td>
<td>0.46</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>$\text{SO}_4^{2-}$ (Met)</td>
<td>5 $\mu$M-SO$_4^{2-}$</td>
<td>2.50</td>
<td>1.72</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>$\text{SO}_4^{2-}$ (Met)</td>
<td>10 mM-SO$_4^{2-}$</td>
<td>11.77</td>
<td>9.97</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>None</td>
<td>1.19</td>
<td>0.34</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>5 $\mu$M-SO$_4^{2-}$</td>
<td>1.60</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>10 mM-SO$_4^{2-}$</td>
<td>2.82</td>
<td>1.66</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>Met (SO$_2^{2-}$)</td>
<td>None</td>
<td>0.86</td>
<td>0.13</td>
<td>0.73</td>
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<tr>
<td></td>
<td>Met (SO$_2^{2-}$)</td>
<td>5 $\mu$M-SO$_2^{2-}$</td>
<td>1.70</td>
<td>0.81</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Met (SO$_2^{2-}$)</td>
<td>10 mM-SO$_2^{2-}$</td>
<td>2.85</td>
<td>1.90</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Met (SO$_2^{2-}$)</td>
<td>0.2 mM-SO$_2^{2-}$</td>
<td>3.83</td>
<td>2.40</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>Met (SO$_2^{2-}$)</td>
<td>20 $\mu$M-S2$^{2-}$</td>
<td>18.66</td>
<td>17.93</td>
<td>0.67</td>
</tr>
<tr>
<td>S30 (met3)</td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>10 mM-SO$_4^{2-}$</td>
<td>1.01</td>
<td>0.08</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>0.2 mM-SO$_2^{2-}$</td>
<td>1.81</td>
<td>0.88</td>
<td>0.93</td>
</tr>
<tr>
<td>STX4-4D (met14)</td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>10 mM-SO$_2^{2-}$</td>
<td>2.41</td>
<td>0.27</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>Met+ $\text{SO}_4^{2-}$</td>
<td>0.2 mM-SO$_2^{2-}$</td>
<td>4.18</td>
<td>2.04</td>
<td>2.14</td>
</tr>
</tbody>
</table>

the absence of sulphate, showed that no $^{64}$Cu eluted in the void volume (e.g. P$_r$) in either case (data not shown). These results were consistent with the conclusion that the P$_r$-associated $^{64}$Cu represented the exchangeable $^{64}$Cu pool (vide supra).

Metabolic activity of the exchangeable $^{64}$Cu-binding pool

A possible copper metabolic relationship between the non-exchangeable $^{64}$Cu pool associated with protoplasts and the exchangeable pool associated with the cell wall was investigated by designing experiments to detect redistribution of $^{64}$Cu from one to the other of these pools. This was an important test of the suggestion that cell wall-bound copper in the yeast Debaryomyces hansenii was available for subsequent cellular utilization (Wakatsuki et al., 1988). In the first protocol, sulphate-free wild type cells were labelled with $^{64}$Cu; the cells accumulated copper primarily into the non-exchangeable pool (cf. Table 2). The labelled cells were then allowed to double once in media that were either sulphate-free or sulphate-supplemented. The two cultures were then challenged with cold copper and the fraction of $^{64}$Cu retained was determined. The results are given in Table 3 (experiment 1) and show that supplementation with sulphate, which allows for the resumption of sulphide generation, does not serve to mobilize $^{64}$Cu from the intracellular, non-exchangeable to the periplasmic, exchangeable $^{64}$Cu-binding pool. In a companion experiment, wild type strain AS2-2A was labelled in a sulphate-containing medium, washed and then allowed to resume growth in a copper-free, sulphate-replete medium. The fraction of the total cell-associated $^{64}$Cu which was non-exchangeable was determined as the culture passed through two generations. These data are given in Table 3 also (experiment 2) and show that the $^{64}$Cu in this pool fractionally decreased as a function of the total protein as expected for the number of culture doublings. That is, in experiment 2 there was no systematic or significant increase in the total amount of non-exchangeable $^{64}$Cu due to redistribution into the cell of $^{64}$Cu originally associated with the exchangeable pool available [initially 0.75 nmol $^{64}$Cu (mg protein)$^{-1}$, see legend to Table 3] during culture growth in the copper-free medium. In summary, these experiments provided no evidence that copper in one or the other of these pools could
when grown in such media which contain wild type strains of yeast accumulate copper only into a intracellular sulphide generation. This distribution obtains up to different from the results of White in a low molecular mass complex which appears to be copper metallothionein. Some copper can also be bound intracellular space. At this concentration of medium cells or to protoplasts and effluxes from the cell very is not exchangeable with cold copper added to labelled pool which appears to be within the cell, that is, in the uptake is energy-dependent, selective for Cu2+, negligible part of &Cu within the cell was not mobilized for efflux by intracellular sulphide generation.

**Discussion**

Media used commonly for the growth of *S. cerevisiae* contain between 150 and 325 nM-copper as determined by flameless atomic absorption spectrophotometry (Lin & Kosman, 1990). The results shown here indicate that when grown in such media which contain < 1 μM-copper, wild type strains of yeast accumulate copper only into a pool which appears to be within the cell, that is, in the intracellular space. At this concentration of medium Cu2+, the intracellular copper is bound primarily in the soluble fraction to Cu,Zn superoxide dismutase and copper metallothionein. Some copper can also be bound in a low molecular mass complex which appears to be copper–gluthathione. This distribution obtains up to 10 μM-Cu2+ in the medium. This result is somewhat different from the results of White & Gadd (1986) who recovered a larger fraction of intracellular copper in a particulate fraction (referred to as ‘bound’). Intracellular uptake is energy-dependent, selective for Cu2+, negligible at 4 °C, and inhibited by cycloheximide (De Rome & Gadd, 1987; Lin & Kosman, 1990). The 64Cu in this pool is not exchangeable with cold copper added to labelled cells or to protoplasts and effluxes from the cell very slowly, if at all. Similarly, intracellular zinc exhibits little efflux from *S. cerevisiae* (White & Gadd, 1987).

The distribution of copper associated with methionine prototrophs grown or incubated in the presence of sulphate and exposed to medium [Cu2+] = 2.5–20 μM is significantly different in that a large and constant fraction of the Cu2+ which becomes cell-associated is exchangeable with extracellular copper. This exchange process is fast compared to net accumulation, is energy-independent and is unaffected by cycloheximide. It is also selective for Cu2+ since it is neither stimulated nor inhibited by Zn2+ (Lin & Kosman, 1990). Even more importantly, it occurs only with cells which have been pre-exposed to copper, that is copper-naive cells do not exhibit this rapid accumulation of 64Cu2+. This result strongly excludes the possibility that rapid non-specific binding of 64Cu2+ to the cell wall makes a significant contribution to the accumulation of label observed.

This exchangeable pool can be localized to the cell wall in that 64Cu can be chased from the cell wall debris resulting from mechanical disruption or from the supernatant obtained following Zymolyase treatment. The cell wall-associated 64Cu appears in the void volume peak when the Zymolyase supernatant is size-fractionated. Undoubtedly, this peak is heterogeneous. Zymolyase, which is a mixture of β-1,3-glucanase and protease activities (Scott & Schekman, 1980), releases a number of uncharacterized soluble mannoproteins from the cell.
wall at least one of which is > 120 kDa (Pastor et al., 1984). Treatment of the void volume fraction with proteinase K does release a number of low molecular mass species, suggesting it is proteinaceous in part (B. F. Crawford, C.-M. Lin & D. J. Kosman, unpublished observations). However, we have not characterized this fraction electrophoretically and therefore cannot compare it to published electrophoretic profiles of cell wall-associated species (Pastor et al., 1984).

A significant result is that $^{64}$Cu$^{2+}$ does not accumulate significantly in the P$_{r}$ chromatographic fraction when added in vitro to the Zymolyase supernatant derived from copper-naive cells. Further data indicate that the metabolic process which supports the deposition of $^{64}$Cu in the P$_{r}$ pool is the generation of sulphide by the cellular reduction of medium sulphate (or sulphite). However, the data do not unequivocally elucidate the relationship between copper uptake and distribution, and sulphide generation, that is the mechanism of the sulphide-dependent deposition of copper on the cell wall. We propose that this deposition is gratuitous and does not represent an active mechanism of copper detoxification on the basis of the following observations.

First, intracellular, non-exchangeable copper was not mobilized into the exchangeable pool upon resumption of sulphate assimilation (Table 3, Experiment 1). This could indicate that sulphide generation is not an element in the partitioning of copper to the periplasm although it is needed for copper to be deposited there. Second, the amount of non-exchangeable copper in all strains (Met$^+$ or Met$^-$) in all media was relatively insensitive to whether active sulphate assimilation was occurring during $^{64}$Cu uptake (Table 2). This result reasonably indicates again that neither the process of copper uptake nor the partitioning event which targets the newly arrived copper to either the intracellular or cell wall pools depended on concurrent sulphide generation. Third, addition of sulphide (at only 20 $\mu$M) to the uptake buffer of sulphate-free cells resulted in a 10-fold increase in the size of the exchangeable pool (Table 2). A simple explanation for this result is that the added sulphide and copper reacted extracellularly and the cell wall simply provided nucleation sites for the precipitation of the resulting copper sulphides. Fourth, none of the methionine auxotrophs we studied were at all copper-sensitive, indicating that their lack of sulphide generation played little role in the way these strains detoxified the metal.

These results most reasonably show that sulphide generation by $S$. cerevisiae does promote deposition of copper on the cell wall, probably as a copper sulphide (Ashida et al., 1963; Kikuchi, 1965; Pan-Hou & Imura, 1981). However, this deposition does not appear to result from a sulphide-dependent extrusion of copper from the intracellular space. There is some indication that large concentrations of sulphide may serve to entrap medium copper so that it becomes unavailable for uptake (Table 2). Literature data (Kikuchi, 1965) indicate that this level of sulphide (20 $\mu$M) is not obtained in culture as result of cell-dependent sulphate reduction, however.

The results and interpretations presented here are quite different than those published by Wakatsuki et al. (1988). In studies of copper accumulation by the yeast $D$. hansenii, these workers showed that cell wall material could bind copper as Cu(I) in a process in which this material carried out the reduction of Cu(II). They also suggested that this cell wall-reduced and -bound copper was available for subsequent uptake into the cell. As the data shown here indicate, cell walls from $S$. cerevisiae do not have the capacity to bind copper effectively unless the cells were pre-exposed to the metal, and that such binding depends primarily on the cell, not cell wall, generation of a reducing agent. In $S$. cerevisiae this agent appears to be H$_2$S. These comparisons indicate that among yeasts there may be several different mechanisms of metal accumulation; however, some general patterns may exist, such as metal reduction as a step in the overall process (Wakatsuki et al., 1991; Dancis et al., 1992).

Although the $^{64}$Cu associated with the protoplast (intracellular space) is stable to exchange from outside the cell, the copper bound to yeast copper metallothionein and Cu,Zn superoxide dismutase is dynamic in that these sites undergo facile exchange in vivo or in a soluble cell extract. The fact that little exchange into either Cu,Zn superoxide dismutase or copper thiorein in buffer occurs suggests that a cytoplasmic factor(s) facilitates this exchange. This behaviour is consistent with the observations reported by Freedman and his co-workers who suggested that glutathione played an important role in intracellular copper trafficking (Freedman & Peisach, 1989; Freedman et al., 1989). We have no direct evidence that glutathione plays a role yeast copper metabolism, or facilitates the $^{64}$Cu exchange observed. However, our data do indicate that copper-glutathione is present in $S$. cerevisiae. A copper-glutathione complex has been observed in the soluble fraction obtained from Neurospora crassa also (Germann & Lerch, 1987). The precise role of glutathione in fungal copper metabolism remains to be elucidated.

Quantification of the concentration and time dependencies of the partitioning event(s) which leads to the $^{64}$Cu distribution described herein are reported in the following paper (Lin et al., 1993). These kinetic analyses suggest a model for copper uptake and distribution in this yeast.

The experiments elucidating the presence of glutathione and the copper-glutathione complex were performed by Richard Hassett. This work was supported in part by grant RO1 GM46787 from the National Institutes of Health of the Public Health Service of the USA.
References


