Distribution of ^{64}Cu in *Saccharomyces cerevisiae*: kinetic analyses of partitioning

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The cell association of copper in the yeast *Saccharomyces cerevisiae* can involve both binding to the cell wall and the accumulation of copper within the cell. The former process requires the concurrent generation of H_2S by the cell via the reduction of sulphate. The contributions of each of these processes to the uptake of ^{64}Cu by wild type and *met3*-containing (ATP sulphurylase-deficient) strains have been kinetically dissected. The Michaelis constant for uptake ($4\ \mu\text{M}$) is independent of the type of cell association which is occurring, suggesting, although not requiring, that both processes are associated with a common kinetic intermediate. The time dependence of the cell-association of ^{64}Cu also suggests the presence of this intermediate pool of bound copper. The $V_{\max}$ for uptake includes a constant contribution from accumulation of ^{64}Cu within the plasmalemma [$0.1\ \text{nmol min}^{-1} (\text{mg protein})^{-1}$] plus that fraction of the ^{64}Cu within the intermediate pool which diffuses away and is trapped on the cell wall as a metal sulphide. This latter contribution to $V_{\max}$ can be two- to three-times greater than the intracellular uptake depending on the amount and type of sulphur supplementation provided in the $^{64}\text{Cu}^{2+}$ uptake buffer. Both processes are energy-dependent although the sulphide-dependent periplasmic accumulation is somewhat more sensitive to metabolic inhibition. This can be attributed to the ATP required for the activation of sulphate prior to its reduction to the level of sulphite and then sulphide. Periplasmic ^{64}Cu accumulation is strongly inhibited by Zn^{2+} and Ni^{2+} . This inhibition is due to competition for cell-generated sulphide; in the presence of $^{65}\text{Zn}^{2+}$, the decrease in ^{64}Cu bound is quantitatively related to the amount of ^{65}Zn which becomes cell-associated. In contrast, intracellular ^{64}Cu uptake is not inhibited by these two metals (at $50\ \mu\text{M}$) showing that the copper translocation pathway is metal-specific. These observations suggest a model for the way newly arrived copper is handled at the cell membrane and is partitioned for intracellular uptake.

Introduction

In previous studies on the fate of newly arrived (^{64}Cu) in a wild type strain of the yeast, *Saccharomyces cerevisiae*, we have shown that the kinetics of uptake and distribution of this copper depended on the copper status of the cells (Lin & Kosman, 1990). Cultures of this methionine prototroph grown in a nutritionally normal, sulphate-containing but copper-free medium ($[\text{Cu}] \leq 15\ \text{nM}$) exhibited a linear uptake of ^{64}Cu . A variety of uptake data indicated that copper accumulation by these copper-naïve cells was saturable, energy-dependent and relatively selective for Cu^{2+} . These results confirmed earlier work by De Rome & Gadd in this yeast (1987).

Subsequent kinetic, cell fractionation, metabolic and genetic studies showed that accumulation of copper by

yeast was more complex than these initial results suggested (De Rome & Gadd, 1987; Lin & Kosman, 1990; Lin *et al.*, 1993). For example, the time-dependence of cellular accumulation of ^{64}Cu in cultures which were copper-supplemented or preincubated with Cu^{2+} ($10\ \mu\text{M}$) prior to initiation of ^{64}Cu uptake was biphasic. The linear uptake as above was preceded by a rapid phase of cell-association of ^{64}Cu ($t_{1/2} \approx 1\ \text{min}$) due to isotope exchange with cell-associated, unlabelled copper. The distribution studies showed that this exchangeable copper was bound to the cell wall. The metabolic and genetic studies demonstrated that this copper was probably present as some form of CuS (Ashida *et al.*, 1963; Kikuchi, 1965) since the appearance of this copper pool required the concurrent cell-dependent generation of sulphide. The data suggested a model of copper deposition on the cell wall in which this sulphide trapped copper before it entered the cell. An important finding was that the intracellular, non-exchangeable ^{64}Cu was not mobilized

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into the periplasmic pool by cellular sulphide generation (Lin *et al.*, 1993).

In this paper, we have evaluated the uptake kinetics in a wild type strain in the absence of sulphate and in a *met3*-carrying strain which is blocked in the activation by ATP of sulphate, which precedes its reduction to sulphide (Masselot & Surdin-Kerjan, 1977; Jones & Fink, 1982). These analyses are necessary to distinguish periplasmic deposition from actual trans-membrane accumulation into intracellular, metabolically active copper stores. These studies are needed to develop a more complete kinetic scheme and pathway for the partitioning of newly arrived copper in *S. cerevisiae*. Also, this paper focuses on the time and concentration dependence of the conversion of copper-naïve cells to copper-treated ones with respect to the appearance of the exchangeable ^{64}Cu pool in the periplasm. Since this conversion occurs at $[\text{Cu}^{2+}]_{\text{medium}} \approx 1 \mu\text{M}$ and greater, this copper metabolic pathway is operable at copper concentrations that can be found in some literature media (Difco Manual, 1984, 10th edn, pp. 1135–1141) and thus represents a fundamental aspect of normal yeast metabolism. Although the cellular response to elevated levels of copper and other transition metals has been studied in some detail in yeast and other micro-organisms (Kikuchi, 1965; Gadd & White, 1985; White & Gadd, 1986; Phelan *et al.*, 1990; Greco *et al.*, 1990), cellular copper handling by yeasts at normal copper levels has been characterized in somewhat less mechanistic detail (Wakatsuki *et al.*, 1979, 1988; Gadd *et al.*, 1984; De Rome & Gadd, 1987). The sulphide-dependent, periplasmic accumulation of copper could be easily dismissed as an artefact. However, it is important to recognize that since true wild type yeasts are prototrophs and thus sulphate assimilators (sulphide generators), they will be handling environmental copper in precisely the manner described in this work. In this report we suggest a working model for copper handling in *S. cerevisiae* which we hope will provide the necessary experimental framework for further studies on copper metabolism in this organism.

Methods

The strains, media, labelling protocols and analytical procedures are described in the preceding paper (Lin *et al.*, 1993). The kinetic data obtained in the work described herein were all fitted by non-linear regression analysis to the appropriate kinetic equation using the program ENZFITTER (Biosoft). For example, for the Michaelis–Menten equation, this program provides standard deviations for the fitted constants, V_{max} and K_m , calculated velocities for each $[S]$, and the residual sum of squares. The kinetic constants and their standard deviations reported derived from a single fit of all data from three separate experiments, each experiment done in triplicate. The mean sum of squares reflected the experimental variance only indicating an adequate fit to this equation. Comparisons of $^{64}\text{Cu}^{2+}$ uptake velocities under different conditions were made using a two-tailed Student's *t*

analysis of mean values as indicated in the legend and footnote to Tables 2 and 3, respectively. These statistical analyses were provided by the program INSTAT (GraphPad).

Results

^{64}Cu uptake by sulphate-depleted and sulphate-supplemented cultures of wild type strain AS2-2A and by a sulphate assimilation mutant, S30

A sulphate-supplemented culture of wild type strain AS2-2A accumulates $2.50 \text{ nmol } ^{64}\text{Cu} (\text{mg protein})^{-1}$ per 30 min, whereas a sulphate-depleted culture accumulates 0.86 nmol (Lin *et al.*, 1993). To explore the mechanism underlying this difference in accumulation of ^{64}Cu explicitly, ^{64}Cu uptake by such cultures was determined and is shown in Fig. 1. The data show that the linear uptake of ^{64}Cu by sulphate-supplemented culture was 2.3-fold that of the sulphate-depleted culture as predicted by the net accumulation measured previously.

The role of sulphate (and sulphide) in the kinetics of cell-association of ^{64}Cu was investigated further. First, with the wild type strain AS2-2A, either the sulphate concentration in the uptake buffer was increased from $5 \mu\text{M}$ to 10 mM , or sulphite (0.2 mM) was added; the uptake velocities of these cells (which were grown in the absence of sulphate) in these two uptake mixtures at $10 \mu\text{M } ^{64}\text{Cu} \cdot \text{His}_2$ were increased from 0.126 to 0.236 and $0.312 \text{ nmol } ^{64}\text{Cu min}^{-1} (\text{mg protein})^{-1}$ respectively. As reported previously, with 10 mM -sulphate or 0.2 mM -sulphite in the uptake buffer, sulphate-depleted AS2-2A accumulated 2.85 and $3.83 \text{ nmol } ^{64}\text{Cu} (\text{mg protein})^{-1}$ per

Fig. 1. Time-dependent cell association of $^{64}\text{Cu}^{2+}$ in wild type and *met3* mutant strains of *S. cerevisiae* grown in sulphate-depleted or sulphate-supplemented media. Cultures were grown and prepared for $^{64}\text{Cu}^{2+}$ uptake as described by Lin *et al.* (1993). Cellular accumulation of $^{64}\text{Cu}^{2+}$ was measured in the presence of $10 \mu\text{M}$ - $^{64}\text{Cu} \cdot \text{His}_2$ with or without $5 \mu\text{M}$ -sulphate at 30°C . The samples were: wild type strain AS2-2A with $5 \mu\text{M}$ -sulphate (○); wild type strain AS2-2A without sulphate (■); and mutant strain S30 (*met3*, ATP sulphurylase-deficient) with or without sulphate (not significantly different, □). The SEM ($n = 4$) for the data points were between 6 and 15%.

Table 1. Kinetic constants for ^{64}Cu uptake by wild type and mutant strains of *S. cerevisiae*

Wild type strain AS2-2A and *met3*-containing mutant, S30 (ATP sulphurylase-deficient) were grown to mid-exponential phase (1×10^7 – 1.5×10^7 cells ml^{-1}) in a copper-free (≤ 15 nM) minimal medium with the sulphate supplementation as indicated. Cells were washed and resuspended in MES-glucose uptake buffer; ^{64}Cu was present as $^{64}\text{Cu} \cdot \text{His}_2$ or $^{64}\text{Cu}(\text{H}_2\text{O})_6^{2+}$ as noted. The kinetic constants given in the table were derived from a direct non-linear fit of initial velocities of uptake versus $[^{64}\text{Cu}]$ using the computer program ENZFITTER.

Strain, conditions and substrate	$V_{\max}$ [nmol ^{64}Cu min^{-1} (mg protein) $^{-1}$]	K_m (μM)
AS2-2A (wild type)		
Sulphate-depleted (+ Met)		
$^{64}\text{Cu} \cdot \text{His}_2$	0.100 ± 0.005	3.8 ± 0.3
Sulphate-supplemented		
$^{64}\text{Cu} \cdot \text{His}_2$	0.210 ± 0.007	4.4 ± 0.4
$^{64}\text{Cu}(\text{H}_2\text{O})_6^{2+}$	$0.243 \pm 0.007^*$	$5.1 \pm 0.4^*$
S30 (<i>met3</i>)		
Sulphate-depleted or supplemented (+ Met)		
$^{64}\text{Cu} \cdot \text{His}_2$	0.103 ± 0.004	2.6 ± 0.3
$^{64}\text{Cu}(\text{H}_2\text{O})_6^{2+}$	0.116 ± 0.005	2.5 ± 0.2

* Values from Lin & Kosman (1990).

30 min, respectively, or 1.7- and 2.3-fold more than in the presence of 5 μM -sulphate (Lin *et al.*, 1993). These data demonstrate the expected positive correlation between the initial velocity of ^{64}Cu accumulation and the net ^{64}Cu accumulated as a function of cellular sulphide generation.

Second, a mutant strain, S30, was used; this strain carries a defect at the *MET3* locus which codes for ATP sulphurylase, the enzyme which activates sulphate for its subsequent reduction to sulphite and then sulphide (Massetot & Surdin-Kerjan, 1977). The time dependence of $^{64}\text{Cu}^{2+}$ uptake by this strain was determined at 10 μM - $^{64}\text{Cu} \cdot \text{His}_2$ (Fig. 1, $\square$). This uptake was independent of the presence of sulphate (see legend). The time-dependent uptake of ^{64}Cu by S30 was similar to that of the sulphate-depleted culture of wild type strain AS2-2A ($\blacksquare$) and was 40% of the uptake by the sulphate-supplemented one ($\circ$). Based on a cellular volume of 60 μm^3 (Stevens, 1982), at $t = 90$ min, strain S30 'concentrated' the ^{64}Cu about 25-fold relative to the medium. This contrasts to the 100-fold 'concentration' of $^{64}\text{Cu}^{2+}$ by a sulphate-supplemented wild type strain. This difference represents the ^{64}Cu accumulation in the periplasmic, exchangeable pool in the latter case (Lin *et al.*, 1993).

Thus, the kinetic data are consistent with the distribution profile of cell-associated ^{64}Cu in that increased sulphide generation, which supports increased total ^{64}Cu accumulation, also supports a quantitatively equivalent increase in the initial velocity of ^{64}Cu uptake. This is true whether the sulphide generation is altered by supplementation with sulphide precursors or by genetic backgrounds which impair enzymic steps upstream from

this metabolic intermediate in sulphate assimilation. The data also support a model in which the increase in cell-association of copper which correlates to sulphide is due entirely to increased accumulation in the periplasmic, exchangeable pool of ^{64}Cu .

Kinetic analyses of ^{64}Cu uptake by sulphate-depleted and sulphate-supplemented cultures of wild type *S. cerevisiae* and by mutant strain S30

The initial velocities of ^{64}Cu accumulation from $^{64}\text{Cu} \cdot \text{His}_2$ by sulphate-depleted and sulphate-supplemented cultures of AS2-2A as a function of $[\text{Cu}^{2+}]$ were determined. The kinetic constants derived from a non-linear fit of these data to the Michaelis–Menten equation are given in Table 1. Kinetic constants for ^{64}Cu uptake from $^{64}\text{Cu}(\text{H}_2\text{O})_6^{2+}$ are given also (Lin & Kosman, 1990).

The importance of these kinetic constants derives from the fact that the sulphate-supplemented culture partitions and accumulates ^{64}Cu into both the periplasmic, exchangeable as well as the intracellular, non-exchangeable pool, whereas the sulphate-depleted culture accumulates ^{64}Cu into the non-exchangeable pool only. The difference in $V_{\max}$ values reflects this fact. Since the fitted K_m value for ^{64}Cu uptake appears sulphate-independent, the accumulation of ^{64}Cu into these two pools may be mediated by the same component(s) in the very early stages of ^{64}Cu uptake. Significant also is the fact that the state of ligation of the Cu^{2+} does not markedly alter the kinetic constants for uptake in sulphide-generating cultures (cf. lines 2 and 3, Table 1). However, this uptake represents a majority (70%) contribution of ^{64}Cu in the

periplasmic, exchangeable pool but only a 30% contribution by ^{64}Cu in the intracellular one. Thus, a specific effect of histidine on ^{64}Cu uptake into the latter, minority pool might be obscured in these data.

The uptake kinetics of the *Met⁻* strain (*met3*) confirmed, however, that intracellular accumulation of ^{64}Cu from the hexaquo Cu(II) ion was kinetically indistinguishable from the uptake from $\text{Cu(II)} \cdot \text{His}_2$ (Table 1). Thus, copper appears to enter the intracellular pool as the free ion and not complexed to an extracellular ligand. Note too the close similarity of V_{max} values for the two strains under comparable conditions (e.g. no sulphide generation) consistent with the suggestion that the dependence of V_{max} on $[\text{sulphate}]_{\text{medium}}$ for strain AS2-2A was due to the level of sulphide generation.

Effects of carbon source and metabolic inhibitors on ^{64}Cu uptake by mutant strain S30

We have shown that ATP is required to sustain net ^{64}Cu accumulation by sulphate-supplemented wild type strain AS2-2A (Lin & Kosman, 1990). ATP is required for the activation of sulphate prior to its reduction (Masselot & Surdin-Kerjan, 1977) and could be necessary for the active transport of copper across the plasmalemma (De Rome & Gadd, 1987; Lin & Kosman, 1990). To assess the relative ATP requirements for the sulphide-dependent, periplasmic ^{64}Cu cell association and intracellular copper accumulation, the effects of carbon source and metabolic inhibitors on ^{64}Cu uptake by the *Met* auxotroph S30 were determined (Table 2). Glucose depletion caused an 84% inhibition of uptake; uptake did not require glucose (glycolysis) specifically, however, since glycerol/ethanol supported ^{64}Cu accumulation (74% of the glucose control). The metabolic inhibitors azide and DNP inhibited uptake by 74 and 87%, respectively, with glycerol/ethanol as carbon sources. These results confirm that ATP, and, perhaps, a transmembrane Δp (Borst-Pauwels, 1981; White & Gadd, 1987) is required to sustain ^{64}Cu incorporation into the intracellular, non-exchangeable pool.

Effects of cycloheximide and other metals on ^{64}Cu uptake by mutant strain S30

The effects of cycloheximide and other metals on the ^{64}Cu uptake velocity of the *met3*-containing strain S30 were determined. This was done to demonstrate directly the role of protein synthesis in intracellular (transmembrane) copper uptake and the metal-specificity of this uptake. Treatment with cycloheximide resulted in a 50% reduction in the uptake velocity at $10 \mu\text{M}$ - $^{64}\text{Cu} \cdot \text{His}_2$ with respect to the no cycloheximide control (Table 3). The concentration of cycloheximide used here was that which

inhibited protein synthesis completely. Note that cycloheximide inhibits total (intracellular plus periplasmic) ^{64}Cu uptake by sulphide-generating cultures > 85% (Lin & Kosman, 1990).

The intracellular uptake process appeared very selective for copper since $50 \mu\text{M}$ - Zn^{2+} or $-\text{Ni}^{2+}$ did not significantly inhibit ^{64}Cu uptake into the intracellular pool in strain S30 (Table 3). This was in contrast to the 50% inhibition of total ^{64}Cu accumulation by a sulphate-supplemented wild type strain, which represented accumulation into both the periplasmic and intracellular pools (Lin & Kosman, 1990). That this difference in inhibition was due to competition by Zn^{2+} with Cu^{2+} in the periplasmic, exchangeable pool was demonstrated by two experiments. First, the distribution of ^{64}Cu accumulated in 30 min by wild type strain AS2-2A in the presence of $50 \mu\text{M}$ - Zn^{2+} (at $10 \mu\text{M}$ - $^{64}\text{Cu} \cdot \text{His}_2$ and $5 \mu\text{M}$ -sulphate) was determined. The Zn^{2+} reduced net ^{64}Cu accumulation from 2.57 to 1.31 nmol ^{64}Cu (mg protein) $^{-1}$. This decrease (-1.26 nmol ^{64}Cu) could be accounted for quantitatively by a reduction in the periplasmic, sulphate-dependent pool of exchangeable ^{64}Cu from 1.63 to 0.35 nmol ^{64}Cu (mg protein) $^{-1}$ (-1.28 nmol ^{64}Cu). This decrease represented an 80% inhibition of ^{64}Cu accumulation in the exchangeable pool.

In a second experiment (data not shown), the accumulation and exchange of ^{65}Zn was investigated in sulphate-supplemented cultures of AS2-2A, grown in either the absence or presence of *Met* (which suppresses sulphide generation), and in cultures of mutant strain S30. *Met* repressed ^{65}Zn accumulation by AS2-2A by 50%, a decrease which correlated to an 80% decrease in an exchangeable pool of ^{65}Zn (cells challenged with $20 \mu\text{M}$ -cold Zn^{2+}). Consistent with this result, the ^{65}Zn accumulated by mutant strain S30 was entirely non-exchangeable; the amount of this non-exchangeable ^{65}Zn was equivalent to the amount of ^{65}Zn accumulated by AS2-2A into the non-exchangeable pool. These results strongly suggest that Zn^{2+} from the medium, like Cu^{2+} , can be entrapped on the cell wall of *S. cerevisiae* into an exchangeable pool, and that the two metals can compete for the cell component(s), probably H_2S , involved in this deposition.

Kinetic analysis of the partitioning of newly arrived ^{64}Cu

To determine if the concentration of $^{64}\text{Cu}^{2+}$ used to label the cells or the concentration of cold Cu^{2+} used in the chase quantitatively altered the distribution of newly arrived ^{64}Cu , and thus provide kinetic evidence for a specific distributive pathway, two experiments were performed. In the first experiment, cells were labelled at various medium $^{64}\text{Cu} \cdot \text{His}_2$ concentrations but chased with a constant $[\text{Cu}^{2+}]$ of $10 \mu\text{M}$ (Fig. 2a). The data

Table 2. *The effects of metabolic inhibitors on $^{64}\text{Cu}^{2+}$ uptake in met3 mutant strain S30*

Met⁻ strain S30 (*met3*, ATP sulphurylase-deficient) was grown in sulphate-depleted medium containing either glucose or glycerol/ethanol to OD₆₆₀ = 1.0. After washing, cells were resuspended in MES-glucose or MES-glycerol/ethanol buffer to the original cell density. $^{64}\text{Cu}^{2+}$ uptake was carried out in the presence of 10 μM - $^{64}\text{Cu}(\text{NO}_3)_2$. Control cells were incubated with carbon source prior to the uptake experiment. For the no carbon source experiment, glucose-grown cells were resuspended in MES buffer alone and incubated for 1 h prior to addition of $^{64}\text{Cu}^{2+}$. As indicated, glycerol/ethanol-grown cultures were pre-incubated with sodium azide or DNP for 1 h; the final concentrations of azide and DNP were 210 and 4 μM , respectively (see Results). Values are means $\pm$ SEM ($n = 4$).

Addition	Carbon source		^{64}Cu accumulation [nmol ^{64}Cu (mg protein) ⁻¹ per 30 min]
	Glucose	Glycerol/ethanol	
None (control)	+		0.70 $\pm$ 0.02
None	None	None	0.11 $\pm$ 0.02*
None (control)		+	0.52 $\pm$ 0.01
Azide		+	0.135 $\pm$ 0.05*
DNP		+	0.07 $\pm$ 0.03*

* These values are different from the respective control values (glucose or glycerol/ethanol, no additions) at $P < 0.01$ using a two-tailed Student's *t* analysis of mean values.

Table 3. *Effect of cycloheximide and metal ions on $^{64}\text{Cu}^{2+}$ uptake velocity of met3 mutant strain S30*

Met⁻ strain S30 (*met3*, ATP sulphurylase-deficient) was grown to OD₆₆₀ = 0.8–1.2. In the cycloheximide experiment, cycloheximide (100 $\mu\text{g ml}^{-1}$) was added for either 20 min or 3 h. Cells were then washed five times with MES-glucose buffer and resuspended in the same buffer (no cycloheximide) to OD₆₆₀ = 1.0. Uptake was initiated by the addition of 10 μM - $^{64}\text{Cu} \cdot \text{His}_2$. The initial velocity was determined by the difference of cell-associated ^{64}Cu between two time points ($t = 0$ and 3 min). In the metal ion inhibition experiments, either Zn²⁺ or Ni²⁺ was added simultaneously (to 50 μM) with $^{64}\text{Cu} \cdot \text{His}_2$ to initiate uptake (no pretreatment with cycloheximide).

Additions to growth medium	Additions to uptake buffer	Initial velocity [nmol ^{64}Cu min ⁻¹ (mg protein) ⁻¹]	Percentage inhibition
None	None	0.069 $\pm$ 0.002	
Cycloheximide (20 min)	None	0.036 $\pm$ 0.002	50
Cycloheximide (3 h)	None	0.030 $\pm$ 0.002	45
None	Ni ²⁺	0.065 $\pm$ 0.003*	0
None	Zn ²⁺	0.064 $\pm$ 0.002*	0

* Not different from control (no additions) using a two-tailed Student's *t* analysis of mean values.

showed that after 30 min labelling at medium [$^{64}\text{Cu}^{2+}$] = 1 μM , the exchangeable pool represented 18% of the total ^{64}Cu accumulation, whereas at [$^{64}\text{Cu}^{2+}$] $\geq$ 2.5 μM , this pool was constant at 74 $\pm$ 4% of the initial cell-associated ^{64}Cu . Previous data showed that at medium [Cu^{2+}] = 15 nM, there was little detectable exchangeable ^{64}Cu (Lin & Kosman, 1990; Lin *et al.*, 1993). In the second experiment, cells were labelled at 10 μM - $^{64}\text{Cu} \cdot \text{His}_2$ ([sulphate] = 5 μM) but the washed cultures were chased with various Cu^{2+} concentrations (Fig. 2*b*). In this case, the results showed that at all chase concentrations of Cu^{2+} the fraction retained was 30 $\pm$ 4% although the velocity of exchange was reduced at 1 and 2.5 μM -

unlabelled Cu^{2+} . This result indicated that the retained fraction of ^{64}Cu after a cold copper chase was independent of the Cu^{2+} concentration used in the chase. The K_m value for copper-naïve, sulphate-supplemented cells was 4.4 μM (Table 1), thus the initial rate of uptake during the $^{64}\text{Cu}^{2+}$ pulse was expected to be directly proportional to [Cu^{2+}] between 1 and 5 μM . Thus, not only the fraction retained (Fig. 2*a*) but also the total pool size, was changing. These results suggest that ^{64}Cu enters a non-exchangeable pool first and fills it to some extent before ^{64}Cu can become associated with the exchangeable one. Based on these data this non-exchangeable pool could be the same or different from the one which

Fig. 2. Copper concentration dependencies of the exchangeable copper-binding pool. Wild type strain AS2-2A was grown in a synthetic, sulphate-supplemented, copper-free medium (Lin *et al.*, 1993), washed and resuspended in MES-glucose buffer containing $5\text{ }\mu\text{M}$ -sulphate. In (a), the samples were labelled for 30 min with 1 (●), 2.5 (○), 5 (□) or 10 (+) μM - $^{64}\text{Cu}\cdot\text{His}_2$, washed, resuspended in the uptake buffer and challenged with cold $\text{Cu}\cdot\text{His}_2$ ($10\text{ }\mu\text{M}$). In (b), samples were labelled for 30 min with $^{64}\text{Cu}\cdot\text{His}_2$ ($10\text{ }\mu\text{M}$), washed, resuspended in the uptake buffer; the initial cell-associated ^{64}Cu was $3.02 \pm 0.09\text{ nmol }^{64}\text{Cu}(\text{mg protein})^{-1}$. Individual samples were then challenged with cold $\text{Cu}\cdot\text{His}_2$ at the following concentrations (μM): 1.0 (●); 2.5 (○); 5.0 (□); 10.0 (+); or 20.0 (△). The values represent means derived from two separate experiments, each involving duplicate samples. The SEM ranged from 6–12%.

represents the 25% of total ^{64}Cu characterized as non-exchangeable in the limit (see below).

The time-dependence of the fate of newly arrived ^{64}Cu was investigated to kinetically characterize this putative intermediate copper-binding pool (Fig. 3). In this experiment, wild type strain AS2-2A was grown in the copper-free, sulphate-containing medium and then incubated with $10\text{ }\mu\text{M}$ - $^{64}\text{Cu}\cdot\text{His}_2$ in MES-glucose buffer for the time period as indicated (see legend) prior to washing and exposure to a $10\text{ }\mu\text{M}$ -cold Cu^{2+} chase. The initial fast ^{64}Cu accumulation associated with the apparent exchangeable pool(s) has been shown to occur only in sulphate-supplemented, Met prototrophs pre-exposed to copper (Lin & Kosman, 1990; Lin *et al.*, 1993). The data demonstrate a well-resolved time dependence in the appearance of this exchangeable pool (Fig. 3a). The data show that the fraction of the total cell-associated ^{64}Cu

Fig. 3. Time dependence of the appearance of the exchangeable ^{64}Cu -binding pool. Wild type strain AS2-2A was grown in a synthetic, sulphate-supplemented, copper-free medium (Lin *et al.*, 1993), washed and resuspended in MES-glucose buffer containing $5\text{ }\mu\text{M}$ -sulphate. Samples were labelled for varying lengths of time with $^{64}\text{Cu}\cdot\text{His}_2$ ($10\text{ }\mu\text{M}$), washed, resuspended in the uptake buffer and challenged with cold $\text{Cu}\cdot\text{His}_2$ ($10\text{ }\mu\text{M}$). The fraction of the initial ^{64}Cu retained was determined as a function of duration of the chase. In (a), the labelling times were: 30 s (○); 1 min (●); 2.5 min (□); 10 min (■). The values for the initial cell-associated ^{64}Cu [$\text{nmol }^{64}\text{Cu}(\text{mg protein})^{-1}$] were: 30 s, 0.079 ± 0.008 ; 1 min, 0.14 ± 0.01 ; 2.5 min, 0.3 ± 0.02 ; 10 min, 1.22 ± 0.06 . In (b), the fraction of the initial ^{64}Cu retained after a 30 min chase is plotted against the labelling time. In (c), the data from (b) are replotted on a semi-log scale. The line represents a non-linear fit of the data to a single exponential using the computer program ENZFITTER with $k = 0.3 \pm 0.04\text{ min}^{-1}$ ($t_{1/2} = 2.3\text{ min}$). The individual values represent means derived from three separate experiments, each involving duplicate samples.

which could be exchanged reached a limiting value at 10 min of labelling (Fig. 3b). These results are consistent with the inference above that when $^{64}\text{Cu}^{2+}$ is present at

Table 4. Kinetic dissection of ^{64}Cu uptake into the intracellular and extracellular pools

Data are for wild type strain AS2-2A (Met prototroph) grown in sulphate-supplemented media.

Specific medium condition or uptake addition	Intracellular uptake [Percentage of glucose control (percentage inhibition)*]	Total uptake [Percentage of glucose control (percentage inhibition, extracellular uptake)†]
+ Glucose	100	100
– Glucose	16 (84)	3 (100)
+ Glycerol/ethanol	74 (26)	56 (51)
+ DNP	26 (74)	23 (78)
+ Azide	13 (87)	20 (77)
+ Cycloheximide	50 (50)	18 (96)
+ Zn^{2+} or Ni^{2+} (50 μM)	100 (0)	50 (71)
Kinetic constants		
V_{max} [nmol copper min^{-1} (mg protein) $^{-1}$]	0.10	0.21
K_m (μM)	3.8	4.4

* Values in parentheses are percentage inhibition of accumulation into the intracellular pool only.

† Values in parentheses are percentage inhibition into the periplasmic pool only, calculated on the basis that approximately 30 % of the total ^{64}Cu accumulation is into the intracellular pool (at 10 μM - ^{64}Cu), corrected for the percentage inhibition into that pool as indicated by the data in the first column.

10 μM in the buffer, an initial step might be required before newly arrived ^{64}Cu appears in the exchangeable pool. The data suggest a model in which newly arrived ^{64}Cu accumulated first in an intermediate, but non-exchangeable pool before it subsequently partitioned into the two types of detectable, end-point copper-binding pools, one exchangeable, the other non-exchangeable. This partitioning appeared to follow first-order kinetics (Fig. 3*b*). Indeed, these data could be fit by a single exponential with $k = 0.3 \text{ min}^{-1}$ as shown in Fig. 3(*c*).

Discussion

The properties of and kinetic constants for ^{64}Cu accumulation into the two cellular copper-binding pools in wild type, sulphate-supplemented *S. cerevisiae* are summarized in Table 4. This comparison suggests the following conclusions about copper accumulation in this yeast. First, in H_2S -generating cultures and at [copper] > 1 μM , copper uptake is dominated by the ATP-dependent reduction of sulphate. This is demonstrated by the partitioning results presented in the preceding paper (Lin *et al.*, 1993) and the kinetic results shown here. This is indicated also by the stronger inhibition of sulphide-dependent, periplasmic ^{64}Cu accumulation by energy depletion or limitation in comparison to the effects of such treatments on intracellular uptake of the metal (Table 4). That the metabolic inhibitors DNP and azide inhibit the two processes to the same extent is not contradictory to this conclusion. Rather, the effects of these compounds can be taken as evidence that intracellular uptake requires a DNP-sensitive H^+ (cation) gradient (Borst-Pauwels, 1981; De Rome & Gadd, 1987;

White & Gadd, 1987) and/or an azide-sensitive metal reduction.

The effect of Zn^{2+} or Ni^{2+} also distinguishes these two processes; the importance of dissecting copper uptake as done here is well-illustrated by the difference in inhibition by either metal on intracellular only versus total uptake. The data show clearly that the pathway for copper uptake into the cell is specific for copper; it is inhibited weakly by Zn^{2+} if at all. This fact is obscured when inhibition of copper 'uptake' by Zn^{2+} is assayed in cells which are actively generating H_2S . Indeed, Zn^{2+} is an excellent competitor for the cell-generated sulphide and thus is an effective inhibitor of copper accumulation in the periplasmic pool.

Cycloheximide treatment resulted in a > 85 % reduction in the $^{64}\text{Cu}^{2+}$ uptake velocity in the sulphate-supplemented wild type strain AS2-2A (Lin & Kosman, 1990). In this strain, 70 % of the total cell-associated ^{64}Cu is partitioned into the periplasmic pool, whereas 30 % is delivered into the intracellular pool. In contrast, cycloheximide caused only a 50 % reduction in $^{64}\text{Cu}^{2+}$ uptake by the *met3*-containing strain S30 (Table 4). This difference indicates that out of the observed 85 % inhibition by cycloheximide seen overall in the *MET3* wild type sulphate-supplemented cells, the reduction of intracellular ^{64}Cu accumulation would have accounted for only 15 % (50 % inhibition of the 30 % of the total ^{64}Cu which was intracellular). The remaining 70 % reduction in uptake must be due to the essentially complete inhibition by cycloheximide of the sulphide-dependent, periplasmic ^{64}Cu accumulation. Thus, this ^{64}Cu accumulation appears more sensitive to cycloheximide than does intracellular ^{64}Cu accumulation. The extreme cycloheximide sensitivity of this pool may

indicate that one or more of the enzymes involved in the activation and reduction of sulphate (Masselot & Surdin-Kerjan, 1977) has a limited half life ($t_{1/2} \leq 4$ min). Alternatively, cycloheximide could have a more direct effect on the components in this pathway. Unfortunately, information relevant to either possibility does not appear in the literature.

The kinetic data indicate that copper enters the cell as the free ion; fractionation and chromatographic results show, however, that it does not exist as a 'free' copper ion after entering the cell (Lin *et al.*, 1993). The lack of copper efflux shows this strongly also (White & Gadd, 1986; Lin & Kosman, 1990; Lin *et al.*, 1993). Thus, intracellular copper is not in thermodynamic equilibrium with respect to the medium. This consideration implies that the demonstrated energy requirement for the intracellular accumulation process is not associated with the maintenance of a copper concentration gradient. It is most likely involved in the uptake process *per se*, perhaps associated with the maintenance of a counter-cation gradient, as noted above (Gadd *et al.*, 1984; De Rome & Gadd, 1987).

The comparative kinetic analyses of intracellular versus total ^{64}Cu uptake is, overall, most informative about the way medium Cu^{2+} interacts with the yeast cell. The $V_{\max}$ value observed for Met wild type, sulphate-supplemented cells represents intracellular ^{64}Cu accumulation plus that fraction of ^{64}Cu which was sulphide-trapped. The intrinsic $V_{\max}$ value of the single, kinetically distinguishable, saturable process may be larger than the one measured, since this value may be limited by the amount of sulphide generated. Thus, the amount of copper which interacts with the saturable element and then diffuses back into the periplasm (ultimately the medium) may be much larger than the amount which is actually measured as being trapped by sulphide. This inference is supported by the fact that the uptake velocity of the wild type culture increased as [sulphate] was increased. Addition of sulphide promotes the accumulation of even more ^{64}Cu , > 95% of which is cell wall-associated and exchangeable (Lin *et al.*, 1993).

The fitted K_m value is independent of the state of ligation of the Cu^{2+} . Also, the uptake data can be fitted to a single kinetic (binding) isotherm – a single K_m value – irrespective of whether intracellular uptake only or both periplasmic and intracellular uptake is being measured. The K_m values determined here are similar to the K_m , $1.1 \mu\text{M}$, determined for the Met prototroph (sulphide-generating) strain x2180 (De Rome & Gadd, 1987). The fit to a single isotherm can indicate that the periplasmic, H_2S -dependent, exchangeable copper pool derives from the same saturable process as does the intracellular copper pool, but does not prove it. Fitting the data to an equation describing two binding isotherms showed that

Fig. 4. Model for the processing of newly arrived ^{64}Cu in *S. cerevisiae*. The numbers in the model refer to the following steps in this pathway. (1) Reduction of Cu(II) to Cu(I) by a membrane metal reductase; the change in redox state promotes the release of the ^{64}Cu from the presenting ligand (L). (2) The metal associates with a membrane component in a kinetically detectable process (K_m). (3) The Cu(I) is transferred to an intermediate copper-binding pool, $k = 0.3 \text{ min}^{-1}$. The ^{64}Cu in this pool is subsequently partitioned between (4) translocation [limiting $V_{\max} = 0.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] and (5) simple diffusion away from the cell surface into the periplasm where it can encounter H_2S and be trapped in the cell wall as Cu_2S .

no strong discrimination between the two fits could be demonstrated for two K_m values differing by less than 2.5-fold (data not shown). Within this limitation, these facts together suggest the following model of Cu^{2+} uptake in *S. cerevisiae*, illustrated in Fig. 4.

(i) A kinetically undetectable ligand dissociation step (step 1) occurs prior to the interaction of the 'free' copper with the primary membrane component associated with kinetic saturation (step 2). This ligand dissociation is indicated by the fact that the kinetic constants are the same for $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ and $\text{Cu} \cdot \text{His}_2$. Reduction of the liganded Cu(II) to Cu(I) by a membrane metal reductase is a reasonable driving force for this ligand displacement. Such reductases are well-known in yeasts (Crane *et al.*, 1982; Lesuisse *et al.*, 1990; Wakatsuki *et al.*, 1991) and are involved in the uptake of Fe(III) (Lesuisse *et al.*, 1987; Lesuisse & Labbe, 1989; Dancis *et al.*, 1990, 1992). As the kinetic data here

indicate for Cu(II) uptake, Fe(III) reduction, although required, does not appear rate-limiting for uptake of Fe^{3+} from the medium (Dancis *et al.*, 1990).

(ii) Since the K_m value for sulphate-supplemented cells appears to be the same as that for sulphate-depleted cells, copper perhaps as Cu(I), interacts with this primary membrane component before it is partitioned into intracellular (non-exchangeable) and periplasmic (exchangeable) accumulation processes (steps 4 and 5, respectively). Neither Zn^{2+} nor Ni^{2+} inhibit this interaction or the partitioning event.

(iii) In the absence of sulphide generation, the copper partitioning into the periplasmic accumulation process simply diffuses back into the extracellular medium since the $V_{\max}$ value is lower in this condition.

(iv) In the presence of sulphide, the Cu^{+2+} diffusing away from the membrane uptake system interacts with sulphide in the periplasm. The resulting CuS is deposited there into the exchangeable pool on the cell wall. Zn^{2+} can compete with the copper for the sulphide at this step.

(v) The observed Cu^{2+} concentration and time dependencies in the appearance of the sulphide-dependent, exchangeable ^{64}Cu pool (Figs 2 and 3) suggest two mechanistic possibilities. First, these observations may indicate simply that the copper in this pathway must accumulate to some threshold level before precipitation of CuS can occur in or on the cell wall (Kikuchi, 1965). Alternatively, they could suggest a more complex model in which downstream from the saturable component (step 3) a small, intermediate, non-exchangeable pool exists from which (excess) Cu^{+2+} diffuses away (step 5) without entering the intracellular uptake pathway (step 4). This 'excess', or, as noted, some fraction of it, subsequently interacts with sulphide.

This model (Fig. 4) suggests that the velocity of translocation of the copper across the plasma membrane (step 4) is comparable to the $V_{\max}$ associated with the saturable component as measured in Met auxotrophs, or in any strain in the absence of sulphate [$0.1 \text{ nmol Cu min}^{-1} (\text{mg protein})^{-1}$]. Indeed, this velocity may be that $V_{\max}$. Kinetically, this model is comparable to that associated with an enzymic double-displacement mechanism (first half-reaction, $k = 0.3 \text{ min}^{-1}$, step 3) in which the catalytic, enzymic intermediate (read 'pool') is partitioned between two alternative acceptors in a system which is rate-limited at the partitioning step (second half-reaction, $V_{\max}$ for the translocation pathway, step 4) (Fersht, 1985). Confirmation of this model at the molecular level will require identification of the cellular components associated with the four steps proposed here: Cu(II) reduction; Cu(I) binding (K_m); binding in an intermediate fraction; and translocation. Characterization of these components is the focus of continuing investigation.

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