Yeast Contain a Non-proteinaceous Pool of Copper in the Mitochondrial Matrix

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Copper is an essential cell nutrient acting as a cofactor in nearly 20 enzymes (1). However, excess accumulation of copper ions results in toxicity. Evidence for the effectiveness of copper ions as a toxin comes from its historic use as a fungicide, molluscide, and algicide. Homeostatic mechanisms exist in cells to regulate the cellular concentration of copper ions, thus maintaining copper balance and minimizing deleterious effects. Cells appear to maintain a quota for essential metal ions; this quota is primarily the quantity necessary to metallate the various copper proteins (2, 3). Copper ions are required for at least three key enzymes in Saccharomyces cerevisiae (4, 17–19). In the absence of CCS copper ions are not inserted into Sod2 in yeast requiring the function of the Lys7 (CCS) metallochaperone (20, 21). Deletion of either Ccc2 or Atx1 results in suppression of growth defects of sod2Δ cells. However, in the absence of CCS within the matrix, the activation of human Sod1 can be achieved by the addition of copper salts to the growth medium.

The mechanism of copper ion routing to the mitochondrion is shown to contain a pool of copper that is distinct from that associated with the two known mitochondrial copper enzymes, superoxide dismutase (Sod1) and cytochrome c oxidase (CcO) and the copper-binding CcO assembly proteins Cox11, Cox17, and Sco1. Only a small fraction of mitochondrial copper is associated with these cuproproteins. The bulk of the remainder is localized within the matrix as a soluble, anionic, low molecular weight complex. The identity of the matrix copper ligand is unknown, but the bulk of the matrix copper fraction is not protein-bound. The mitochondrial copper pool is dynamic, responding to changes in the cytosolic copper level. The addition of copper salts to the growth medium leads to an increase in mitochondrial copper, yet the expansion of this matrix pool does not induce any respiratory defects. The matrix copper pool is accessible to a heterologous cuproenzyme. Co-localization of human Sod1 and the metallochaperone CCS within the mitochondrial matrix results in suppression of growth defects of sod2Δ cells. However, in the absence of CCS within the matrix, the activation of human Sod1 can be achieved by the addition of copper salts to the growth medium.

Copper is from copper in Sod1, Fet3, and CcO. Expression of these copper-binding proteins varies with growth conditions, suggesting that the copper may be distributed differently depending on growth conditions. Clearly, a significant fraction of the cellular copper is associated with Sod1; however, not all Sod1 molecules are metallated (4, 5). Fet3 requires four copper ions for activity, but levels of this protein are dependent on iron status of the medium (6). CcO levels vary depending on whether the cells are grown by fermentation or respiration. In addition, a varying quantity of cellular copper exists bound to two metallothioneins, Cup1 and Crs5 (7, 8). Expression of CUP1 and CRS5 is regulated by copper levels through the copper-responsive transcription factor Ace1 (9, 10). An increase in the free Cu(I) ion pool activates Ace1 through formation of a polyprotein (11). Activated Ace1 induces transcription of CUP1, CRS5, and SOD1. Each protein is capable of buffering the copper ion concentration in the cytoplasm. Copper buffering regulated by Ace1 must be highly efficient because yeast cells are predicted to lack a pool of free copper ions in the cytoplasm (4).

Cells concentrate copper by several orders of magnitude from the culture medium to achieve the copper quota (2). This gradient is generated by multiple metal ion permeases on the plasma membrane. Copper ion uptake is mediated by high affinity Ctr1 and Ctr3 and low affinity Smf1 and Fet4 permeases (12). Within the cell other transporters are necessary for transmembrane movement of copper ions. Translocation of copper ions into the lumen of trans-Golgi vesicles is achieved by P-type ATPase transporters (Ccc2 in yeast, ATP7A and ATP7B in animal cells) (13). Translocation of copper ions across the vacuolar membrane is mediated by Ctr2 (14).

Copper ions are shuttled to sites of utilization by protein-mediated transfer (15, 16). Metallochaperones facilitate the transport of copper ions to specific sites through a chelating environment and prevent the inappropriate interaction of copper ions with other molecules. Copper insertion into Sod1 in yeast requires the function of the Lys7 (CCS) metallochaperone (4, 17–19). In the absence of CCS copper ions are not inserted into yeast Sod1 unless cells are incubated in medium containing high levels of copper salts, and this requirement is attenuated by a decrease in levels of the metallothioneins by a deletion of ACE1 (4). This is an indication of the cytosolic copper-buffering capacity of Cup1. Copper insertion into Fet3 occurs within post-Golgi vesicles. The copper is translocated across the vesicular membrane by the Ccc2 P-type ATPase (13). Cu(I) ions are shuttled to the Ccc2 translocase by the Atx1 metallochaperone (20, 21). Deletion of either Ccc2 or Atx1 results in apo-Fet3 and an inability to grow on iron-deficient medium (20). Cu(I) ions translocated by the Ccc2 pump can also be used to metallate heterologous molecules within trans-Golgi vesicles (22).

The mechanism of copper ion routing to the mitochondrion...
for assembly of cytochrome c oxidase is unknown. Because copper ion delivery to Sod1 and Ccc2 is protein-mediated, the prediction is that copper shuttling to the mitochondrial will also be protein-mediated. Two proteins implicated in copper ion translocation to the mitochondrion are Cox17 and Cox19 (23–25). Both proteins are conserved in eukaryotic cells and exhibit a dual localization in both the cytosol and the mitochondrial intermembrane space (IMS) (24, 25). The IMS is an aqueous space between the mitochondrial inner membrane (IM) and outer membrane (OM) and is interrupted by junction points in which the IM and OM are in contact (26). Cox17 has been implicated in copper ion delivery to the mitochondria based on its dual localization and the fact that the respiratory defect of cox17–1 cells is suppressed by high levels of copper in the growth medium (23). A copper transport function for Cox17 is shown because it binds Cu(I) through three critical cysteinyl residues in a Cys-Cys-Xaa-Cys sequence motif (27). Cox19 shows weak sequence similarity to Cox17 in the conservation of three C-terminal Cys residues (25); however, these Cys residues in Cox17 are not part of the Cu(I) binding motif (27). Yeast cells lacking Cox17 or Cox19 are respiratory-deficient and devoid of cytochrome c oxidase activity. Suppression of these phenotypes by the addition of exogenous copper is observed only in cox17 cells.

Within the mitochondrial two additional inner membrane proteins, Sco1 and Cox11, are implicated in copper ion insertion into cytochrome c oxidase (28–33). Sco1 and Cox11 are important in reactions of direct copper transfer to the CuA site and CuB site, respectively. The two copper centers exist within mitochondrially encoded subunits of cytochrome c oxidase, so copper ions must be shuttled to the mitochondrion for insertion into nascent chains within the inner membrane. Cox17 may deliver Cu(I) to both Sco1 and Cox11 for subsequent donation into nascent chains within the IMS (25). A W303 based cox17 strain (W303: MAT a, ade2-1, his3-1,15, leu2-3,112, trp1-1, ura3-1, Δcox17:TRP1), obtained from Dr. Alexander Tzagoloff, and its isogenic wild-type strain were used for all Cox17 experiments. BY4741 strains deleted for ACE1, SOD2, LYS7, and CCC2 were purchased from Research Genetics. Cells lacking Sod1 were obtained from Dr. Jerry Kaplan. Cells lacking Cox2 were provided by Drs. R. Poyton and T. Fox. The cox17/cox19 double null mutant was isolated by deleting COX19 in a W303 cox17 strain using KanMX.

Expression Vector—Human SOD1 was obtained from Dr. P. J. Hart and inserted into TcPhs316 and fused to the 5' 27 codons of the yeast SOD2 ORF that encode the mitochondrial target sequence. The fusion gene, designated SOD2/hSOD1, was under the control of the SOD2 promoter with the hSOD1 terminator. A variant of this fusion gene was constructed in which only the promoter of SOD2 was fused to hSOD1. This fusion, designated hSod1(1c), lacked the mitochondrial target sequence of Sod1 and gave cytosolic expression. These constructs were confirmed by dideoxyribonucleotide sequencing. CCS-encoding plasmids pLS114 and pLS117 were generously provided by Dr. Val Culotta. Both contain CCS (LYS7) fused to segments of CYB2 in pRS313. Plasmid plS117 encodes a fusion of the CYB2 IMS-targeting sequence to LYS7 (37), whereas pLS114 encodes a chimera of 80 codons of CYB2 fused to LYS7 that targets CCS to the matrix. Plasmids were transformed into yeast by the lithium acetate protocol.

Preparation of Mitochondria—Mitochondria were isolated as described previously (38). Briefly, lysate was used to create spheroplasts that were gently ruptured in a glass Dounce homogenizer. After the spheroplasts were lysed crude mitochondria were isolated by differential centrifugation in the presence of 0.5 mM phenylmethylsulfonyl fluoride. Cell debris was pelleted at 1,500 × g, mitochondria and microsomes were pelleted at 12,000 × g; the supernatant represented the post-mitochondrial fraction. The crude mitochondria were then loaded onto a discontinuous Nycodenz gradient (16% on 22%) and centrifuged at 150,000 × g for 1 h. The intact mitochondria recovered from the gradient interface were washed in isotonic buffer and pelleted at 12,000 × g before analysis. Mitochondria were quantified using standard Bradford protein reagents. For hypotonic lysis, the intact mitochondria were diluted 20-fold into 10 mM Hepes, pH 7.4, and centrifuged at 25,000 × g to isolate the soluble IMS. The pellet was retained as mitoplasts and later fractionated into soluble and membrane fractions by sonication or carbonate treatment in 100 mM NaCO3, pH 11.

Mitochondrial Matrix Copper Pool

Mitochondrial Fractionation—Mitochondria were isolated by deleting COX17 or COX19, and also digested in the acid-washed tubes for comparison, and spiked with commercially available mixed metal standards were used to construct a standard curve. Blanks of nitric acid or buffer samples were also digested in the acid-washed tubes for comparison, and spiked controls were analyzed to ensure reproducibility.

Cytochrome c Oxidase Assays—Cytochrome c oxidase activity in isolated mitochondria was quantified by monitoring the reduction of 32 ppb bovine cytochrome c at 550 nm by 5–10 μg of isolated mitochondria (40 mM KH2PO4, pH 6.7, 0.5% Tween 20).

Mitochondrial Fractionation—Purified mitochondria were lysed by three 30-s pulses of sonication at 50% output of a microtip. The soluble fraction was isolated from the insoluble fraction by centrifugation at 15,000 × g. The soluble fraction was diluted into buffer A (20 mM

V. Culotta, personal communication.
per-binding protein. If Cox17 and/or Cox19 are dominant co-
demonstrated previously that Cox17 binds Cu(I) within a poly-
addition, both proteins are capable of binding copper ions. We
in both the cytoplasm and the IMS of mitochondria (24). In
nitric acid. The remainder of the sample was used for SDS-PAGE
throughout the run and analyzed by ICP-OES after dilution into 10%
in 20 mM Tris HCl, pH 7.2, 1 M NaCl. Fractions were collected
in a copurifying organelle. Gradient-purified mitochondrial may
contain limited vacuolar and ER contamination (38). Western
analyses were conducted on crude and gradient-purified mito-
chondria to address contamination. Vacular and ER markers
were readily apparent in the crude mitochondrial fraction, but
the marker proteins were significantly reduced in the purified
mitochondrial fraction (Fig. 2A). Despite a marked diminution
in vacuolar and ER markers in gradient-purified mitochondria,
the variance observed in 10 independent
the copper content (nmol of copper/mg of protein) did not ap-
preciably change. The variance observed in 10 independent
gradient purifications was less than 30%, with multiple iso-
lates having a greater copper content in the purified relative to
the crude fraction. To confirm that vacuolar contamination is
not the source of the mitochondrial copper, mitochondria were
purified from vps33 mutant cells that have abnormally small
vacuoles (43). The mitochondrial copper content was un-
changed as was the ratio of copper to heme A, confirming that

RESULTS

Copper Delivery to the Mitochondria—Cox17 and Cox19 have
been implicated as mitochondrial copper ion shuttles for as-
sembly of cytochrome c oxidase based on their dual localization
in both the cytoplasm and the IMS of mitochondria (24). In
addition, both proteins are capable of binding copper ions. We

demonstrated previously that Cox17 binds Cu(I) within a poly-
copper-thiolate cluster (39). Recombinant Cox17 is also a copper-binding protein. 3 If Cox17 and/or Cox19 are dominant copper
ion shuttles for assembly of CcO, the prediction is that cells
lacking Cox17 or Cox19 would have depressed mitochondrial
copper levels. The copper levels were measured in Nycodenz-
purified mitochondria from a variety of cells lacking one of the
CcO assembly factors. The amount of copper within the mito-
chondron was independent of the presence of Cox17 or Cox19
(Fig. 1A). Although Cox17 and Cox19 cannot complement each
other, a potential redundant role was investigated by testing a
double cox17,cox19 null strain. As shown in Fig. 1A, the mito-
chondrial copper was not affected by this double deletion. In
fact, the mitochondrial copper concentration was not markedly
diminished in yeast lacking Cox2, which is the mitochondrially
encoded subunit of CeO that forms the CuA site, or in rho-
cells, which are devoid of mitochondrially encoded proteins.
The level of mitochondrial copper was independent of the car-on source used for culturing yeast (data not shown). The lack
of an appreciable diminution in mitochondrial copper in cells
lacking a functional CeO complex suggests that mitochondrion
contain a pool of copper not associated with CeO or CeO assem-
bly proteins.

Non-respiratory Pool of Mitochondrial Copper—To measure
the fraction of mitochondrial copper associated with the CeO
complex, we quantified the heme A pool within mitochondria.
CeO is the only protein to use heme A, and all heme A is
associated with the active, oligomeric CeO complex. Yeast mu-
tenants exhibiting defects in assembly of CeO (e.g. cox11, cox17,
cox19, and sco1) are devoid of heme A within the mitochondrion
(23, 30, 40). Using an established extinction coefficient for
heme A +Aα, the amount of CeO can be quantified from the
difference absorption spectrum of oxidized versus reduced cy-
tochromes (41). Because the active CeO binds three copper ions
per complex, the fraction of mitochondrial copper associated
with CeO can be determined. This analysis revealed that ~10%
of the mitochondrial copper was associated with CeO (Fig. 1B).
This calculation is based on the assumption that all CeO mol-
ecules are copper-loaded. This assumption is reasonable be-
cause it is known that mutants that fail to insert copper into
CeO result in an unstable complex that does not contain heme
A (23, 30). The ratio of copper to total protein or heme A was
unaffected by growth in a non-fermentable carbon source. This
is consistent with the deletion mutant data in Fig. 1A showing
that removal of a CeO subunit or several assembly factors has
no significant effect on the copper levels. The known assembly
factors involved in copper ion metallation of CeO are not likely
to contribute to the mitochondrial copper pool because they are
low abundance proteins. A recent mitochondrial proteomic
study showed Sco1 to be a low abundant protein comparable in
levels of the CeO subunit Cox6 (42).

The copper pool is associated with the mitochondrion and not
a copurifying organelle. Gradient-purified mitochondrial may
contain limited vacuolar and ER contamination (38). Western
analyses were conducted on crude and gradient-purified mito-
chondria from various respiratory mutants and their iso-
genic wild-type (WT). Mitochondria were acid-digested, and total met-
als were quantified by ICP-OES. The copper content is expressed per
mg of mitochondrial protein. Panel B, fraction of mitochondrial copper
associated with CeO. CeO was quantified in purified mitochondria
based on heme A content. Heme A was quantified from the oxidized
versus reduced difference absorption spectra in mitochondria solubili-
ized in 0.5% deoxycholate. The spectra represent the dithionite-re-
duced spectrum minus the ferricyanide-oxidized spectrum. An extinct-
ion coefficient of 14 mol−1 cm−1 for A605 nm − (A520 nm − A620 nm)2
was used for heme A quantitation. The samples were then analyzed by
ICP to quantify the copper concentrations. A ratio of 3 copper per 2
heme A was used to determine the percentage of copper that is heme
A-associated.

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3 K. Rigby, unpublished observation.
the copper did not arise from vacuolar contamination (Fig. 2B).

To confirm that ER/Golgi vesicle contamination was not a concern, mitochondria isolated from ccc2 null cells were analyzed. Ccc2 is the P-type ATPase copper transporter that is responsible for translocation of Cu(I) into post-Golgi vesicles. The mitochondrial copper content in ccc2 cells was similar to that of wild-type cells (Fig. 2B).

**The Mitochondrial Copper Pool Is Not Associated with Known Yeast Copper Proteins**—The only two known copper enzymes within mitochondria are CcO and Sod1. A fraction of the cytosolic Cu,Zn Sod1 partitions in the mitochondrial IMS (36). The CCS metallochaperone responsible for copper insertion into Sod1 is involved in the recruitment of Sod1 to the IMS (36). Cells lacking CCS have diminished levels of Sod1 within the IMS. We observed that deletion of LYS7 encoding CCS does not significantly diminish the mitochondrial copper pool (Fig. 3). Two other cytosolic copper-binding proteins include the Cup1 and Crs5 metallothioneins. Both proteins bind multiple Cu(I) ions within polycopper clusters (8). Distribution of these molecules within the mitochondrial matrix may contribute a significant quantity of mitochondrial copper. Expression of *CUP1* and *CRS5* is regulated by the copper-responsive transcriptional activator Ace1. Deletion of *ACE1* results in a marked decrease in basal *CUP1* and *CRS5* expression in yeast (44). Cup1 and Crs5 do not contribute to the mitochondrial copper pool because ace1Δ cells have wild-type levels of mitochondrial copper (Fig. 3). Similarly, *crs5Δ* cells have at least wild-type levels of mitochondrial copper. The only other known cuproenzymes in yeast are the plasma membrane Fet3 and vacuolar Fet5 ferroxidases (45, 46). Neither of these is expected to contaminate the copper pool in gradient-purified mitochondria. In addition, the mitochondrial copper pool is unperturbed in cells cultured in high iron-containing medium in which Fet3 and Fet5 are slowly expressed. The mitochondrial copper pool is not attenuated by overexpressing cytoplasmic copper ion buffers. Cells expressing *CUP1* from the constitutive *ADH1* promoter on a high copy plasmid did not diminish the level of mitochondrial copper (data not shown). Likewise, overexpression of human *SOD1*, which increased markedly the copper binding Sod1 levels in the cytoplasm, did not attenuate the mitochondrial copper pool (data not shown).

**Localization of the Copper Pool**—The mitochondria has three distinct compartments, the IMS, the intercristal space, and the matrix. Incubation of mitochondria with hypotonic solution results in rupture of the OM and solubilization of proteins localized within the IMS and presumably intercristal space. OM fragments are retained with the mitoplasts. Incubation of yeast mitochondria in hypotonic buffer results in retention of the bulk of mitochondrial copper with the mitoplast fraction (Fig. 4A). Western blot analysis confirmed that endogenous Sod1 was released by the hypotonic treatment consistent with its localization within the IMS (Fig. 4B). As expected, the known matrix protein Sod2 was retained with the mitoplasts. The marked release of Sod1 in hypotonic-treated mitochondria yet retention of the bulk of the copper with mitoplasts is consistent with the data with *lys7Δ* cells showing that Sod1 does not contribute significantly to the mitochondrial copper pool.

To address whether copper was associated with the membrane fraction or soluble matrix compartment, mitochondria were subjected to sonication to disrupt both membranes. Analysis of the resulting membrane fraction and soluble fraction revealed that the soluble fraction contained little CuO activity but a majority of the copper and the soluble IMS protein Cyb2 (Fig. 5, A and B). The membrane fraction contained the bulk of the CuO enzymatic activity and the OM protein porin (Por1). These results suggest that the bulk of the mitochondrial copper exists within the matrix compartment. This matrix compartmentalization of copper is unprecedented, with no known copper-enzymes present in the matrix. Because the IM forms a permeability barrier to ions, the matrix copper pool must require transport across the IM.

**Copper Pool Is Dynamic**—To determine how the mitochondrial pool of copper responds to changes in copper levels in the growth medium, cells were cultured in synthetic medium containing 0.5 mM CuSO4. It was known previously that yeast incubated in copper-supplemented medium take up additional copper well beyond the copper quota. *CUP1* expression is dramatically up-regulated in response to the increased cellular copper levels, and a significant fraction of the increased cellular copper is retained in the cytoplasm as CuCup1 complexes (47). We show presently that mitochondria also accumulate a fraction of the increased cellular copper. The mitochondrial copper pool is increased 5–10-fold in cells cultured with 0.5 mM CuSO4. The accumulated mitochondrial copper has no negative effect.
on respiration as measured by oxygen consumption. Under culture conditions with 0.5 mM CuSO₄, much of the cytoplasmic copper is associated with the Cup1 and Crs5 metallothioneins (8). The effect of these copper-buffering molecules can be assessed by evaluating ace1Δ cells with attenuated basal and induced expression of CUP1 and CRS5. Because ace1Δ cells are sensitive to elevated copper concentrations (10), cells were incubated with 0.01 mM CuSO₄ for only 5 h. Under these conditions mitochondria from wild-type cells show only a modest increase in copper (2.3-fold) (Fig. 6). The bulk of the increased copper (2.3-fold) (Fig. 6). The bulk of the increased copper accumulation in total cellular copper. These data suggest that zinc and manganese may compete with copper for mitochondrial uptake.

Analyses of Mitochondrial Copper—The avid uptake of copper and the lack of known copper enzymes in the matrix suggest that additional cuproproteins may exist. After sonication of purified mitochondria the soluble fraction was loaded onto a Mono Q anion exchange column. The bulk of the copper was retained and eluted with application of a salt gradient (Fig. 7, panel A). The anionic character of the copper pool yields significant purification from mitochondrial proteins, as many matrix proteins are cationic. The copper-containing fraction quantified by ICP-OES was further fractionated by gel filtration chromatography (Fig. 7, panel B). The anionic copper pool chromatographed in fractions corresponding to the elution volume of a globular protein of 13 kDa. This fraction was, however, devoid of absorbance at 280 nm (data not shown). Repeated attempts to identify proteins in the copper-containing fraction by liquid chromatography-MS/MS after trypsin digestion or electrospray ionization-MS analysis for intact polypeptides resulted in no identifiable proteins. In contrast, a different column fraction containing manganese (Fig. 7, dashed line) was similarly analyzed and found to contain MnSod2 by both liquid chromatography-MS/MS for a tryptic digest and electrospray ionization-MS for the intact protein. Additionally, gel filtration fractions containing the copper pool failed to show any visible protein bands using Sypro-Ruby staining of SDS-PAGE gels. The detection limit of Sypro-Ruby staining is 2 ng.

To further probe the possibility of a copper-binding polypeptide, the copper-pool eluant from Mono Q was subjected to proteinase K digestion. However, devoid of absorbance at 280 nm (data not shown). Repeated attempts to identify proteins in the copper-containing fraction by liquid chromatography-MS/MS after trypsin digestion or electrospray ionization-MS analysis for intact polypeptides resulted in no identifiable proteins. In contrast, a different column fraction containing manganese (Fig. 7, dashed line) was similarly analyzed and found to contain MnSod2 by both liquid chromatography-MS/MS for a tryptic digest and electrospray ionization-MS for the intact protein. Additionally, gel filtration fractions containing the copper pool failed to show any visible protein bands using Sypro-Ruby staining of SDS-PAGE gels. The detection limit of Sypro-Ruby staining is 2 ng.

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were analyzed for metal content. Size exclusion standards ovalbumin, elution fractions 8 and 10 from Mono Q were individually Panel B—fractions is shown as the percent recovered. The salt gradient is shown.

addition the profile does not change if the soluble fraction is prepared by detergent extraction or detergents are added after sonication.

Chromatographic analyses were also conducted with purified mitochondria isolated from ace1Δ, cox17Δ cells and wild-type cells cultured with added additional CuSO4. The elution profile of the copper pool were equivalent in each case (data not shown). Mitochondria from cells cultured with added copper showed a marked increase in the quantity of the anionic copper pool. The similar elution profile of this pool from Mono Q to that shown). Mitochondria from cells cultured with added copper showed no stained band. A polypeptide present at 1

matrix copper pool is accessible to heterologous molecules, human Sod1 was targeted to the mitochondrial matrix in an sod2Δ strain. Appending the mitochondrial matrix targeting sequence of yeast SOD2 to human SOD1 resulted in the import of hSod1 into the mitochondria (Fig. 9A) and mitoplasts (Fig. 9B). The sod2Δ strain is sensitive to oxidative stress particularly under conditions of hyperoxia (48, 49). The SOD2/hSOD1 chimera (designated Sod1(m)) for matrix hSod1 in Fig. 10) was able to suppress the hyperoxia growth impairment of sod2Δ cells only if cells were cultured on agar plates containing 0.1 mM CuSO4 (Fig. 10A). The sod2Δ cells are also impaired in glycerol growth. This phenotype was partially suppressed by SOD2/hSOD1, but glycerol growth was improved with the addition of 0.1 mM CuSO4 (Fig. 10B). To ensure that the rescue of the sod2Δ cell phenotype was due to matrix hSod1 and not trace levels of cytosolic hSod1, we expressed hSod1 under the yeast SOD2 promoter but lacking the Sod2 mitochondrial target sequence. The resultant hSod1 was no longer localized in the matrix (Fig. 9B). No growth of sod2Δ cells harboring the hSOD1 (designated Sod1(c) for cytosolic Sod1 in Fig. 10) was observed under hyperoxia conditions or on glycerol plates regardless of the amount of exogenous copper added to the cultures (Fig. 10, A and B). The lack of suppression of sod2Δ cells by hSOD1(c) is consistent with the observation that the

![Figure 7](http://www.jbc.org/)

**Fig. 7. Chromatography of the soluble mitochondrial fraction after brief sonication.** Panel A, the soluble mitochondrial fraction was fractionated by Mono Q anion exchange chromatography. Elution fractions were analyzed by ICP-OES to establish metal profiles for copper (solid line) and manganese (dashed line). Metal quantitation of fractions is shown as the percent recovered. The salt gradient is shown. Panel B, elution fractions 8 and 10 from Mono Q were individually loaded onto a size exclusion column (Superdex 75), and the fractions were analyzed for metal content. Size exclusion standards ovalbumin, trypsin, and RNase elute in fractions 10, 12, 14, respectively.

![Figure 8](http://www.jbc.org/)

**Fig. 8. Elution behavior of the anionic matrix copper pool with (solid line) and without (dashed line) incubation with proteinase K (panel A).** The Mono Q elution fraction 10 from Fig. 7 was divided into two fractions that were rechromatographed on Mono Q after dilution of the salt content. One sample was incubated with proteinase K (PK) overnight at 23 °C before rechromatography. The peak fraction after chromatography was concentrated, and the retentate was analyzed on a 15% SDS-PAGE gel stained with Sypro Ruby (panel B). The loss of protein bands in the sample incubated with proteinase K confirms the activity of the proteolytic digestion.

### Table I

**Metal concentration in mitochondria versus whole cells**

|       | Copper | Iron | Zinc | Manganese |
|-------|--------|------|------|-----------|
|       | Mito   | WC   | Mito | WC        | Mito   | WC   | Mito | WC | Mito | WC |
| CM    | 1.3 ± 0.2 | 0.7 ± 0° | 2.3 ± 0 | 4.1 ± 0° | 0.7 ± 0° | 2.3 ± 0° | 0.13 ± 0° | 0.55 ± 0° |
| CM + copper | 8.1 ± 0.3 | 128 ± 5 | 1.4 ± 0° | 3.2 ± 0° | 0.3 ± 0° | 2.7 ± 0° | 0.03 ± 0° | 0.29 ± 0° |
| CM + zinc | 0.6 ± 0° | 1.1 ± 0.3 | 1.5 ± 0.1 | 3.8 ± 0.1 | 3.9 ± 0.3 | 62 ± 3 | 0.06 ± 0° | 0.39 ± 0° |
| CM + iron | 1.4 ± 0.1 | 1.3 ± 0.3 | 13 ± 1 | 31 ± 0.9 | 0.9 ± 0.1 | 2.2 ± 0° | 0.08 ± 0° | 0.32 ± 0° |

* S.D. ≤ 0.05.
Mitochondrial Matrix Copper Pool

DISCUSSION
Mitochondria are presently shown to contain a pool of copper that is distinct from the only two known mitochondrial cuproenzymes, Sod1 and CcO. Only a small fraction of mitochondrial copper is associated with these two cuproenzymes. The bulk of the remainder is localized within the matrix as a soluble, anionic, low molecular weight complex. The mitochondrial copper pool is dynamic, responding to changes in the cytosolic copper level. The addition of copper salts to the growth medium leads to an increase in mitochondrial copper. This increase in mitochondrial copper is more pronounced when Cup1 metallothionein expression is attenuated by deletion of ACE1. The mitochondrial copper pool is not diminished by constitutive expression of CUP1 or SOD1, suggesting that a committed pathway for copper ion uptake into the mitochondrion exists.

The identity of the matrix copper ligand is unknown. Several lines of evidence suggest that the matrix copper fraction is not protein-bound. First, no protein bands were observed in the purified fraction by SDS-electrophoresis and Sypro-Ruby staining. Second, no proteins were observed by mass spectrometry. Third, the elution behavior on anion exchange chromatography did not change after prolonged digestion with proteinase K. The conclusion that the matrix pool is non-proteinaceous rests on the assumption that our isolation protocol for the matrix fraction did not liberate copper ions from a labile protein complex. Exposure to air may liberate copper from an oxygen-labile protein. This situation is unlikely considering copper complexes are reasonable air-stable including the thiolate-rich.

**Fig. 9. Western analyses of human Sod1 expressed in yeast.** Panel A, Western analysis of purified mitochondria isolated from either wild-type (WT) and sod2Δ transfectants expressing Sod2/hSod1. Antisera to human Sod1, yeast Sod2, and yeast porin were used. Panel B, Western analysis of cell extract, purified mitochondria (Mito), or mito-plasts of sod2Δ transfectants expressing human Sod1 either with the Sod2 matrix target sequence (+MTS) or without the Sod2 matrix target sequence (−MTS). Westerns for porin and Pgk1 are included for loading controls.

**Fig. 10. Suppression of growth defects of sod2Δ cells by matrix localized human Sod1.** Panel A, growth of sod2Δ transfectants in hyperoxia versus normoxia. Agar plates prepared with medium containing 0.1 mM CuSO4 or lacking added copper were incubated in a chamber purged with 100% oxygen for hyperoxia growth or cultured in atmospheric oxygen for normoxia growth. Transformants included cells harboring human Sod1 targeted to the matrix (Sod1/m) and cells with hSod1(m) and matrix-targeted CCS (CCS(m)) as well as cells with human Sod1 localized in the cytosol hSod1(c). Panel B, growth of sod2Δ transfectants with glycerol as the carbon source. Cells were plated on either glucose or glycerol-containing agar plates in the presence or absence of 0.1 mM CuSO4, vec, vector control; SC, synthetic complete medium.
metallothioneins. It is unlikely that the pool is copper GSH because gsh1Δ cells cultured in medium containing dithiothreitol, to enable growth, contained the same mitochondrial copper level. Furthermore, the anionic character of this pool from gsh1Δ cells did not change. A number of organic acids (citrate, oxaloacetate, etc.) exist within the matrix and may be ligands. Considering that the mitochondrial copper pool is dynamic and can expand significantly as a similar anionic complex, the prediction is that ligands are not limiting within the matrix. Genetic and biochemical approaches are being taken to identify the matrix copper ligand(s).

The matrix copper pool is accessible. Co-localization of human Sod1 and CCS within the mitochondrial matrix results in suppression of growth defects of sod2Δ cells. In the absence of CCS within the matrix, the activation of hSod1 requires the addition of copper salts to the growth medium. Because mitochondrial import of proteins into the matrix occurs as unfolded molecules, the copper metallation of hSod1 must occur within the matrix. The CCS requirement for efficient activation of Sod1 may arise from its role as a copper metallochaperone in directed copper ion insertion in apo-Sod1 (4). Alternatively, Sod1 may arise from its role as a copper metallochaperone in mitochondrial copper transporter translocates multiple metal ions, the mitochondrial may have a buffering capacity for diverse metal ions. Cells lacking Mtm1 show no Sod2 activity, but mitochondrial Mn(II) levels are normal (54). Thus, mtm1Δ cells are expected to contain a pool of non Sod2-bound Mn(II).

Mitochondria can contain a storage pool for metals besides copper. The pool of zinc in mammalian mitochondria increases with increasing cytosolic zinc (55, 56). Likewise, labile iron levels increase significantly within the mitochondria of yeast mutants defective in iron-sulfur cluster formation and processing (57, 58). The increase in the mitochondrial labile iron pool has deleterious consequences. The elevation in labile iron in mitochondria of cells depleted for Yfh1 or Isa1 results in an increase in petite formation (59, 60). Whereas increases in the labile iron pool in mitochondria are deleterious, a 5–10-fold increase in the matrix copper pool is not associated with mitochondrial DNA damage. The ligands of the copper matrix must reduce the reactivity of this pool.

The significance of the matrix copper pool is unclear. Copper ions are needed for assembly of CuA and CuB sites inCcO as well as metallation of Sod1 within the IMS. A common pathway of copper ion delivery to the IMS may exist to provide copper ions for CuC assembly and metallation of Sod1. Cu(I) delivery to the IMS may occur through a permease within the OM or through a shuttle protein. Alternatively, copper may be delivered to the IMS from the matrix. The matrix copper pool may represent a storage pool of copper available to an IM transporter for delivery to the IMS. Attempts are being made to identify such a transporter.

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