Saccharomyces cerevisiae Vacuole in Zinc Storage and Intracellular Zinc Distribution

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Previous studies of the yeast Saccharomyces cerevisiae indicated that the vacuole is a major site of zinc storage in the cell. However, these studies did not address the absolute level of zinc that was stored in the vacuole nor did they examine the abundances of stored zinc in other compartments of the cell. In this report, we describe an analysis of the cellular distribution of zinc by use of both an organellar fractionation method and an electron-probe X-ray microanalysis. With these methods, we determined that zinc levels in the vacuole vary with zinc status and can rise to almost 100 mM zinc (i.e., 7 x 10^6 atoms of vacular zinc per cell). Moreover, this zinc can be mobilized effectively to supply the needs of as many as eight generations of progeny cells under zinc starvation conditions. While the Zrc1 and Cot1 zinc transporters are essential for zinc uptake into the vacuole under steady-state growth conditions, additional transporters help mediate zinc uptake into the vacuole during “zinc shock,” when zinc-limited cells are resupplied with zinc. In addition, we found that other compartments of the cell do not provide significant stores of zinc. In particular, zinc accumulation in mitochondria is low and is homeostatically regulated independently of vacuolar zinc storage. Finally, we observed a strong correlation between zinc status and the levels of magnesium and phosphorus accumulated in cells. Our results implicate zinc as a major determinant of the ability of the cell to store these other important nutrients.

Zinc is an important structural and/or catalytic cofactor for numerous proteins. For example, it was estimated recently that as many as 10% of the approximately 30,000 different proteins encoded by the human genome require zinc for their function (1). If accumulated in excessive amounts, however, zinc can also be toxic to cells (4). Thus, organisms have evolved with homeostatic mechanisms to maintain a relatively constant intracellular environment in the face of changing levels of extracellular zinc. One primary mechanism of zinc homeostasis is the control of zinc uptake across the plasma membrane (11). In addition, sequestration of zinc within intracellular organelles is an important strategy of zinc homeostasis. Organellar zinc sequestration is clearly needed for zinc homeostasis in fungi and plants and may also be a component of mammalian zinc homeostasis (3, 8, 10, 20, 33, 34, 37, 41).

In the yeast Saccharomyces cerevisiae, approximately 1.5 x 10^7 total zinc atoms per cell are required for optimal growth (34). This value is referred to as the “zinc quota” for yeast and probably represents the level required to optimally metalate zinc-dependent proteins in the cell (40). Below that amount, cells grow more slowly. When zinc levels drop below a minimum threshold of ~5 x 10^6 atoms per cell, growth ceases altogether. Our previous results have shown that the Zap1 transcription factor helps maintain intracellular zinc levels for growth by regulating several genes in response to zinc deficiency (32, 52). These genes include the ZRT1, ZRT2, and FET4 genes encoding zinc uptake transporters on the plasma membrane (47, 50, 51). Increased expression of these genes increases the zinc uptake capacity of the cell more than 100-fold.

Previous studies have implicated the vacuole of yeast as a major site of zinc sequestration in the cell, and the level of zinc in the vacuole is also controlled by Zap1 (5, 20, 34, 48). Two transporters, Zrc1 and Cot1, are responsible for zinc transport into this compartment. Genetic studies of these transporters have indicated three major roles for vacuolar zinc sequestration. First, the vacuole is required for zinc detoxification. When zinc is abundant, the metal is taken up into this compartment, thereby limiting its toxicity. Zrc1 and Cot1 are both required for zinc detoxification. Second, the vacuole provides a site of zinc storage. When cells are zinc replete, Zrc1 and Cot1 mediate zinc accumulation in the vacuole. Under conditions of zinc deficiency, the Zrt3 transporter is up-regulated by Zap1 to mobilize sequestered zinc from that compartment (34). Finally, the vacuole plays an especially important role in the resistance to “zinc shock” (36). Zinc shock occurs when zinc-limited cells are resupplied with zinc. Because zinc-limited cells have such a high capacity for zinc uptake, addition of even modest amounts of zinc results in the accumulation of extremely high concentrations of the metal. The vacuole and the Zrc1 and Cot1 transporters are required for surviving zinc shock.
These genetic studies indicated that vacuolar zinc sequestration was critical for zinc homeostasis. However, several questions remained unanswered. For example, what are the levels of zinc sequestered in the vacuole under various conditions of zinc availability? Second, what is the role of the vacuole in buffering zinc levels in other compartments, such as the cytosol and mitochondria? Third, are there any significant sites of zinc storage elsewhere in the cell? Finally, what is the distribution of other elements in the cell and does zinc status alter these distributions? These important questions were addressed in this study.

**MATERIALS AND METHODS**

Yeast strains and growth conditions. *S. cerevisiae* strains DY1457 (MATa ade6 can1 his3 leu2 trpl ara3) and CM104 (MATa ade6 can1 his3 leu2 trpl ara3 zrc1Δ::His3 cot1Δ::URA3) were used in this study (34). Yeast strains were grown in zinc-replete synthetic defined (SD) medium that contains 0.7 M glutamate (Q-BIOgene) plus 1% (wt/vol) glucose and any necessary auxotrophic requirements. SD medium contains 0.9 mM Ca, 3.5 mM Cl, 1.2 mM Fe, 7 mM K, 4.1 mM Mg, 1.7 mM Na, 7 mM P, and 2.5 mM Zn. Zinc-limited cells were grown in Chelex-treated synthetic defined (CSD) medium (32) with 2% nitrogen base (Q-BIOgene) plus 2% (wt/vol) glucose and any necessary auxotrophic requirements. CSD medium is zinc limiting because zinc was removed from SD medium by pretreatment with Chelex 100 resin (Sigma). The concentration of zinc remaining in CSD medium is less than 100 nM (32), and other divalent cations were added back to their original concentrations. Cells were also grown in low-zinc medium (LZM), which is similar to SD medium but zinc limiting due to the addition of 1 mM EDTA, a strong zinc chelator (16). For steady-state growth experiments, yeast strains were incubated for 20 h with aceration at 30°C in CSD or SD medium supplemented with up to 1,000 μM ZnCl2. CSD medium contains a basal zinc concentration of 2.5 μM. For zinc shock experiments, cells were zinc limited by overnight growth in CSD medium, their cell walls were removed to form spheroplasts, and they were then treated with 100 μM ZnCl2. Spheroplasts were used to allow subsequent organelle isolation. Zinc treatments were performed in media containing 0.6 M sorbitol to maintain the integrity of the spheroplasts. The cell number per milliliter of culture was determined by measuring the optical density at 600 nm (OD600) and comparing with a standard curve. All cells were harvested in mid-log phase (OD600 of 0.5 to 1) before being processed for subsequent analysis. Spheroplasts were counted using a hemocytometer.

**AAS.** Measurements of total cellular zinc accumulation were performed by atomic absorption spectrophotometry (AAS). Cells were harvested by centrifugation for 5 min at 1,000 × g and washed twice in cold 1 mM EDTA and once in cold distilled deionized water to remove surface-bond zinc. Cells were then digested overnight at room temperature in 5 ml 15% H2O2, 40% HNO3 (vol/vol). Acid-digested samples were diluted with distilled deionized water, and zinc content was determined by AAS. The protocol was adapted from R. Elmer (280°C for 10 min). The yield of intact mitochondria was determined by activity assays of the matrix enzyme succinate dehydrogenase (39). Aliquots (200 μl) of isolated mitochondria and solubilized vacuoles were digested with 1 volume of 65% HNO3 (vol/vol) at 90°C overnight prior to ICP-MS.

**Elemental analysis by ICP-MS.** ICP-MS was used to measure Zn levels in isolated organelles. Samples were transferred to Pyrex tubes and digested with 0.5 ml concentrated HNO3 at 110°C for 4 h. Each sample was diluted to 4 to 6 ml with 18 meq/mol water and analyzed with an Elan DRCe ICP-MS instrument (Perkin Elmer). Methane was used as a collision cell gas to measure iron. Gallium and indium were used as internal standards, added to the digestion acid bottle to a concentration of 20 ppb. National Institute of Standards and Technology traceable single-element ICP standards were used to make up the calibration standards.

**Immunoblot analysis.** Whole-cell lysates and lysates of isolated organelles were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting was performed using standard protocols (18). A BM chemiluminescence kit (Roche) was used for detection. Antibodies used were mouse anti-cytochrome c oxidase subunit I (COX; 7A10; 1:10,000 dilution), goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Pierce Chemical Co.).

**Cryopreparation and electron probe X-ray microanalysis (EPXMA).** The cells were centrifuged for 3 min at 1,800 × g in a Sorval RT6000B centrifuge. The supernatant was decanted immediately and the pellet prepared for X-ray microanalysis as described in detail in our previous studies (26–28). For cryopreservation, approximately 5 μl of the pellet was resuspended in 0.6 M sorbitol, 0.5 M phenylmethylsulfonyl fluoride) and centrifuged at 1,500 × g for 5 min. The supernatant was transferred to a polyallomer tube, and washed twice in cold 1 mM EDTA and once in cold distilled deionized water. The pellet was resuspended in 20 ml buffer A (10 mM MES [morpholinethanesulfonic acid]-Tris, pH 6.9, 0.1 M MgCl2, 12% [wt/vol] Ficoll 400) with a Dounce homogenizer and then centrifuged at 60,000 × g for 10 min. The supernatant was transferred to a polyallomer tube, and washed twice in cold 1 mM EDTA and once in cold distilled deionized water. The pellet was resuspended in 20 ml buffer A (10 mM MES-Tris, pH 6.9, 0.5 mM MgCl2, 8% [wt/vol] Ficoll 400), and then centrifuged at 60,000 × g for 30 min. The pellets were again collected, resuspended in 0.2 ml 20% buffer C (10 mM MES-Tris, pH 6.9, 5 mM MgCl2, 25 mM KCl), and stored at −80°C.

**Carboxypeptidase Y (CPY) assays were performed to determine the yield of intact vacuoles (29).** Vacuoles were incubated in solubilization buffer and subjected to 80°C. The yield of intact vacuoles was determined by activity assays of the matrix enzyme succinate dehydrogenase (39). Aliquots (200 μl) of isolated mitochondria and solubilized vacuoles were digested with 1 volume of 65% HNO3 (vol/vol) at 90°C overnight prior to ICP-MS.

**Statistical analyses.** EPXMA data were obtained from 17 to 76 cells for whole-cell measurements, 12 to 44 cells for vacuole measurements, and 17 to 43 cells for nonvacuolar cytoplasm measurements, depending on the strain and growth condition. Separate statistical analyses were done by compartment (whole cell, vacuole, or nonvacuolar cytoplasm). For each element, effects of

Briefly, spheroplasts were homogenized in buffer A (20 mM MES, pH 6.0, 0.6 M sorbitol, 0.5 M phenylmethylsulfonfonyl fluoride) and centrifuged at 1,500 × g for 5 min. The supernatant was collected and centrifuged at 12,000 × g for 10 min. The pellet was resuspended in 0.2 ml buffer A without phenylmethylsulfonfonyl fluoride. Five ml of 19% (wt/vol) Nyoced in buffer A was overlaid with 5 ml of 13.5% (wt/vol) Nyoced in buffer A. The gradient was overlaid with the crude mitochondrion resuspension and centrifuged at 160,000 × g for 30 min. Mitochondria, appearing as a brown band between the 19% and 13.5% (wt/vol) Nyoced layers, were collected, resuspended in 20 ml buffer B (20 mM MES, pH 7.4, 0.6 M sorbitol), and centrifuged at 12,000 × g for 10 min. The pellet was resuspended in buffer C and stored at −80°C. The yield of intact mitochondria was determined by activity assays of the matrix enzyme succinate dehydrogenase (39).
TABLE 1. Effects of zinc status on the accumulation and distribution of zinc

| Strain | Treatment | Zn content (nmol/mg [dry wt]) (mean ± SE) | Compartment | P value |
|--------|-----------|------------------------------------------|-------------|---------|
|        |           | Whole cell | Vacuole | Cytoplasm | L vs M | M vs H | L vs H |
| WT     | L         | ND | 1.4 ± 1.7 | 1.7 ± 1.6 | Whole cell | NS | <0.001 | <0.005 |
|        | M         | ND | ND | ND | Vacuole | NS | <0.0001 | <0.0001 |
|        | H         | 3.8 ± 0.9 | 11.9 ± 1.4 | 0.7 ± 0.9 | Cytoplasm | NS | NS | NS |
|        | 0         | ND | 1.4 ± 1.7 | 1.7 ± 1.6 | Whole cell | <0.0001 | NS | <0.0001 |
|        | 10        | 7.1 ± 1.0 | 18.6 ± 2.4 | 1.4 ± 1.2 | Vacuole | <0.0001 | <0.05 | <0.0001 |
|        | 20        | 9.2 ± 1.7 | 36.4 ± 5.3 | 3.4 ± 1.0 | Cytoplasm | NS | NS | NS |
| zrc1Δ cot1Δ mutant | 0 | ND | 0.2 ± 1.2 | ND | Whole cell | <0.0001 | <0.01 | <0.0001 |
|        | 10        | 1.9 ± 0.6 | 6.5 ± 1.3 | 0.7 ± 0.9 | Vacuole | <0.0001 | <0.001 | <0.0001 |
|        | 20        | 5.6 ± 1.0 | 13.9 ± 2.6 | 1.8 ± 0.7 | Cytoplasm | NS | NS | NS |

The levels of zinc in whole cells, vacuoles, and the nonvacuolar cytoplasm were determined by EPXMA under steady-state (first three rows) and zinc shock (all other rows) conditions. Data were obtained from 17 to 76 cells for whole-cell measurements, 12 to 44 cells for vacuole measurements, and 17 to 43 cells for nonvacuolar cytoplasm measurements, depending on the strain and growth condition. WT, wild type; L, low zinc (CSD medium); M, moderate zinc (SD medium with 0 Zn); H, high zinc (SD medium plus 100 μM Zn); 0, 0 min; 10, 10-min Zn treatment; 20, 10-min Zn treatment plus 20-min chase; ND, not detectable; NS, not significant (P > 0.05).

strains and conditions and possible interactions were determined using an extension of the Mann-Whitney test to two-factor analysis of variance on ranked data. Post hoc examination of pairs of strain levels (Tables 1 and 2) under each treatment condition was done using the Mann-Whitney test. Analysis of covariance allowing for differing slopes was performed on the EPXMA data obtained from individual cells, regressing one element on another and adjusting for compartment, strain, and condition as appropriate. The replicate effect was negligible and not further considered. Type III adjusted P values for Fig. 6 and 7 are reported in Results.

RESULTS

Effect of zinc availability on total cellular and organellar zinc levels. To assess the effects of zinc status on cellular zinc accumulation, wild-type cells were grown in media containing a range of zinc concentrations and then assayed for total zinc levels by AAS. zrc1Δ cot1Δ mutant cells were also assayed to determine whether loss of the Zrc1 and Cot1 vacuolar zinc transporters alters zinc accumulation and the distribution of zinc within cells. As shown in Fig. 1A, low levels of total cellular zinc in wild-type cells grown in zinc-deficient CSD medium were measured (17.8 ± 1.6 pmol/10^7 cells). When wild-type cells were grown in untreated SD medium, containing a basal concentration of 2.5 μM Zn, a higher level of total zinc was observed (57.0 ± 3.0 pmol/10^7 cells). With increasing amounts of ZnCl₂ added to SD medium, total cellular zinc accumulation also increased. The most dramatic increase was observed when cells were grown in SD medium plus 1.000 μM ZnCl₂, suggesting a loss of zinc homeostasis under these high-zinc conditions. Consistent with this conclusion, cell growth is blocked when the metal is added at concentrations greater than 4 mM (36).

zrc1Δ cot1Δ mutant cells also had low total zinc levels when grown in CSD medium (11.1 ± 0.1 pmol/10^7 cells) and increased zinc levels when grown in SD medium supplemented with up to 25 μM ZnCl₂. Because of the lack of the vacuolar zinc transporters in this mutant, these cells are unable to tolerate zinc concentrations above 25 μM (36) and therefore could not be assayed at higher concentrations. However, over the range of zinc availability from CSD medium to SD medium plus 25 μM ZnCl₂, little difference in total accumulation was observed between mutant and wild-type cells. Given the role of Zrc1 and Cot1 in transporting zinc into the vacuole, these results indicate that vacuolar sequestration is not a major factor in determining total zinc accumulation over this range of extracellular zinc. An important role for the vacuole at concentrations higher than 25 μM is suggested by the sensitivity of the mutant to increased zinc levels.

Previous studies indicated that zinc can be sequestered in the vacuole, but the actual zinc storage capacity of the vacuole was not determined (34, 36). To assess the effects of zinc status on vacuolar zinc accumulation, intact vacuoles were isolated from cells grown as described above for Fig. 1A. Zinc content was then determined by ICP-MS because this method has a greater sensitivity for zinc than does AAS. Both analytical methods yielded statistically equivalent results for total zinc accumulation (data not shown), demonstrating that values obtained with these two methods can be compared directly. Immunoblot analysis showed that the isolated vacuoles used in this study were not contaminated by the cytosol or other subcellular compartments (see Fig. S1 in the supplemental material). Specifically, the Dpm1 ER protein, the mitochondrial Cox2 protein, and the cytosolic Pgi1 protein were not detected in isolated vacuole preparations. As expected, the Vma1 vacuolar membrane protein and the CPY luminal vacuolar protein were highly enriched in these samples. The vacuolar zinc measurements were normalized based on the recovery of CPY enzymatic activity to quantify the recovery of intact vacuoles.

The effects of zinc status on vacuolar zinc accumulation in wild-type cells were similar to those observed for total zinc levels (Fig. 1B). Vacuolar zinc in CSD medium-grown wild-type cells was 10.4 ± 3.6 pmol/10^7 cells. Increasing extracellular zinc concentrations resulted in markedly elevated vacuolar zinc accumulation. A large increase in zinc accumulation was observed to occur in vacuoles of cells grown with 1,000 μM zinc, consistent with the loss of homeostatic regulation suggested above for total zinc. Consistent with the expected loss of vacuolar zinc transport, zrc1Δ cot1Δ mutants had very low vacuolar zinc levels at all concentrations tested. For example, vacuolar zinc in mutant cells grown in SD medium with no...
TABLE 2. Effects of zinc status on the accumulation and distribution of other elements in wild-type cells

| Element | Treatment | Whole cell | Vacuole | Cytoplasm | Compartment | L vs M | M vs H | L vs H |
|---------|-----------|------------|---------|-----------|-------------|--------|--------|--------|
| Ca      | L         | 15.8 ± 0.9 | 28.4 ± 2.0 | 12.3 ± 0.9 | Whole cell  | <0.005 | <0.0001 | NS     |
|         | M         | 12.1 ± 0.9 | 18.9 ± 2.2 | 9.9 ± 1.1  | Vacuole     | <0.0001 | <0.001  | <0.05  |
|         | H         | 16.8 ± 0.7 | 23.2 ± 2.1 | 14.2 ± 0.7 | Cytoplasm   | NS     | <0.0001 | NS     |
| Cl      | L         | 62.4 ± 7.7 | 53.9 ± 7.1 | 81.7 ± 15.4 | Whole cell  | NS     | <0.0001 | <0.0001 |
|         | M         | 68.5 ± 6.6 | 56.6 ± 6.2 | 83.1 ± 9.8  | Vacuole     | <0.05   | <0.0001 | <0.0001 |
|         | H         | 11.2 ± 0.6 | 7.8 ± 0.6  | 12.0 ± 0.6  | Cytoplasm   | NS     | <0.0001 | <0.0001 |
| Fe      | L         | 3.0 ± 0.2  | 2.4 ± 0.1  | 2.8 ± 0.2   | Whole cell  | NS     | NS     | NS     |
|         | M         | 4.2 ± 0.7  | 3.5 ± 0.9  | 4.2 ± 1.0   | Vacuole     | NS     | NS     | NS     |
|         | H         | 3.1 ± 0.1  | 2.9 ± 0.1  | 3.1 ± 0.2   | Cytoplasm   | NS     | NS     | NS     |
| K       | L         | 511.6 ± 21.0 | 266.8 ± 20.6 | 664.8 ± 23.5 | Whole cell  | NS     | <0.0001 | <0.0001 |
|         | M         | 557.1 ± 24.2 | 539.6 ± 53.3 | 525.8 ± 26.5 | Vacuole     | <0.0001 | <0.0001 | <0.0001 |
|         | H         | 806.9 ± 23.5 | 752.2 ± 34.9 | 761.1 ± 33.2 | Cytoplasm   | <0.005  | <0.0001 | NS     |
| Mg      | L         | 82.7 ± 9.4  | 236.4 ± 19.0 | 42.7 ± 1.9  | Whole cell  | NS     | <0.0001 | <0.0001 |
|         | M         | 82.3 ± 8.0  | 233.3 ± 17.0 | 46.6 ± 4.6  | Vacuole     | NS     | <0.005  | <0.0001 |
|         | H         | 150.9 ± 11.2 | 320.7 ± 18.9 | 93.5 ± 3.5  | Cytoplasm   | NS     | <0.0001 | <0.0001 |
| Na      | L         | 11.7 ± 6.3  | 16.8 ± 11.9 | 14.9 ± 13.1 | Whole cell  | <0.0001 | <0.0001 | NS     |
|         | M         | ND         | ND         | ND         | Vacuole     | <0.0001 | <0.0001 | <0.0001 |
|         | H         | 9.8 ± 2.6  | 37.4 ± 4.3  | 0.6 ± 2.6   | Cytoplasm   | <0.0001 | <0.0001 | NS     |
| P       | L         | 670.4 ± 49.2 | 1,496.6 ± 68.1 | 447.9 ± 12.6 | Whole cell  | NS     | <0.0001 | <0.0001 |
|         | M         | 645.3 ± 50.9 | 1,422.8 ± 83.8 | 449.9 ± 42.9 | Vacuole     | NS     | <0.0001 | <0.0001 |
|         | H         | 1,084.2 ± 48.6 | 1,812.7 ± 65.1 | 830.3 ± 20.4 | Cytoplasm   | NS     | <0.0001 | <0.0001 |

* The levels of the listed elements in whole cells, vacuoles, and the nonvacuolar cytoplasm were determined by EPXMA under steady-state (first set of elements Ca to P) and zinc shock (second set of elements Ca to P) conditions. Data were obtained from 34 to 76 cells for whole-cell measurements, 17 to 44 cells for vacuole measurements, and 17 to 43 cells for nonvacuolar cytoplasm measurements, depending on the growth condition. See footnote a of Table 1 for abbreviations.

added zinc was 13.4 ± 3.4 pmol/10⁶ cells. These results indicate that no other transporter proteins contribute significantly to vacuolar zinc influx under these steady-state growth conditions.

Zinc accumulation in isolated mitochondria was also assayed. Mitochondria were isolated with their contents intact, as determined by recovery of the matrix enzyme succinate dehydrogenase. While isolated mitochondria were free of detect-
able vacuolar and cytosolic contamination, some ER membranes, as indicated by detection of Dpm1, copurified with the mitochondria (see Fig. S1 in the supplemental material). Because the contents of the highly reticulated structure of the ER are likely to be lost during cell disruption, we believe that the zinc levels measured in these preparations correspond largely to mitochondrial zinc, although we cannot exclude entirely some contribution of ER zinc. Zinc accumulation in the mitochondrial preparations was much lower than the vacuolar levels. The level of mitochondrial zinc in CSD medium-grown wild-type cells was approximately 1 pmol/10⁶ cells and increased to 5 to 10 pmol/10⁶ cells in SD medium-grown cells (Fig. 1C).

Little change in mitochondrial zinc levels occurred in cells grown with up to 100 μM zinc, suggesting this level was regulated perhaps by mitochondrial zinc import and/or export transporters. A marked increase in zinc was again observed at 1,000 μM, suggesting that this homeostatic control was overwhelmed in these cells.

Compared to the wild type, the zrc1Δ cot1Δ mutant showed no significant difference in mitochondrial zinc levels as extracellular concentrations were raised from CSD medium to SD medium containing 25 μM added zinc. Given the absence of vacuolar zinc accumulation observed for the zrc1Δ cot1Δ mutant (Fig. 1B), these data indicate that vacuole zinc sequestration does not influence zinc levels in the mitochondria over this range of zinc. In addition, given that total cellular zinc increases for the mutant over this range (Fig. 1A) while mitochondrial zinc does not, we conclude that excess zinc accumulates in another compartment of zrc1Δ cot1Δ cells, perhaps the cytosol. Attempts to directly analyze cytosolic zinc by this fractionation method were thwarted by breakage of a large fraction of the vacuoles (>50%) and release of their zinc during cytosol preparation (data not shown).

**Analysis of intracellular zinc distribution by EPXMA.** An important caveat of the organellar isolation experiments described above is that some zinc may be lost from intact vacuoles and/or mitochondria by transporter-mediated efflux during their isolation. Therefore, the values of zinc measured in these studies may be underestimates of the true in vivo values. To address this issue, an alternative approach to examine the intracellular distribution of metal ions, EPXMA, was used. Wild-type yeast cells were grown under low-zinc (CSD medium), moderate-zinc (SD medium), and high-zinc (SD medium plus 100 μM ZnCl₂) conditions and then quickly frozen, cryosectioned, freeze-dried, and analyzed by EPXMA. This method preserves the intracellular distribution of labile elements that can be lost in chemically fixed cells. EPXMA also has the advantage of measuring several different elements (e.g., Ca, Cl, Fe, K, Mg, Na, P, and Zn) in the sample simultaneously. We will first focus on the results obtained for zinc and then consider the effects of changing zinc status on the distribution of other elements later in this report. An example analysis of the distributions of various elements detectable by EPXMA is shown in Fig. 2. Figure 2, left, shows STEM images of several cells grown in either low zinc (CSD medium) (Fig. 2A) or high zinc (SD medium plus 100 μM ZnCl₂) (Fig. 2B). Some deformation of these normally spherical cells due to cryosectioning was observed. The other panels show the distributions of Ca, P, and Zn in these cells, represented using a false color temperature scale. Phosphorus and magnesium were used as vacuole markers because these elements are known to accumulate in the vacuole to high levels (2, 24). Total, vacuolar, and nonvacuolar cytoplasmic zinc levels for wild-type cells are reported in Table 1. While these values were below the level of detection in CSD medium- and SD medium-grown cells, zinc was detectable in cells grown in SD medium plus 100 μM ZnCl₂. The level of vacuolar zinc in these cells was at least 10-fold greater than the nonvacuolar cytoplasmic level. Thus, in cells grown in SD medium plus 100 μM ZnCl₂, the great majority of intracellular zinc is sequestered in the vacuole. In addition, the absolute level of vacuolar zinc is similar to that in isolated vacuoles measured by ICP-MS. When converted to units of zinc per cell, EPXMA indicated that vacuolar zinc in cells grown in SD medium plus 100 μM ZnCl₂ was 248 ± 29 pmol/10⁶ cells. These results are in close agreement with the data obtained with isolated vacuoles (183 ± 12 pmol/10⁶ cells) (Fig. 1B). Zinc levels in zrc1Δ cot1Δ mutants were below the level of detection by EPXMA under all growth conditions (data not shown). It should be noted that the sums of the vacuolar and nonvacuolar cytoplasmic zinc levels reported in Table 1 do not equal the whole-cell amount because of slight differences in the dry weights of the compartments with which the values have been normalized.
Capacity of the vacuolar zinc store to sustain cell growth.

The results shown in Fig. 1 and Table 1 indicate that yeast cells grown in high zinc can accumulate a large amount of vacuolar zinc. Based on these data, we estimated that cells grown in SD medium plus 1,000 μM ZnCl₂ accumulate as much as 900 pmol vacuolar zinc/10⁶ cells. This value corresponds to 7 × 10⁸ atoms of vacuolar zinc per cell. Our previous studies indicated that the threshold amount of total intracellular zinc required for cell growth is 5 × 10⁶ atoms of zinc per cell (34). Thus, we predicted that high-zinc-grown cells containing an abundant supply of vacuolar zinc could grow for many cell divisions in a medium completely devoid of available extracellular zinc. For example, we predicted that cells grown in SD medium plus 1,000 μM ZnCl₂ would contain sufficient intracellular zinc to undergo eight subsequent doublings, i.e., over 200 cells would grow as progeny from a single original zinc-loaded cell. To test this hypothesis, cells were pregrown in SD medium supplemented with different levels of zinc to generate increasing levels of vacuolar zinc stores and then reinoculated into LZM with no added zinc. Cell growth, as measured by the optical density of the culture, was monitored over time, and the resulting data were then converted to numbers of population doublings for each condition (Fig. 3A). Wild-type cells grown in SD medium and therefore having relatively low vacuolar zinc underwent only three doublings over a 28-h period in LZM. Three doublings would generate eight progeny cells per initial inoculated cell. Cells pregrown in SD medium plus 25 to 1,000 μM ZnCl₂ showed progressively higher growth yields. As we predicted, cells grown in SD medium plus 1,000 μM ZnCl₂ underwent eight doublings of cell number. Several lines of evidence indicate that these effects are due to mobilization of vacuolar zinc stores. First, zrc1Δ cot1Δ mutants grown in SD...
medium plus 25 μM ZnCl₂ grew only slightly better than SD medium-grown wild-type or mutant cells. In addition, wild-type and zrc1Δ cot1Δ mutant cells grown in SD medium with no added zinc and then inoculated into LCM plus 100 μM ZnCl₂ (Fig. 3A, “+Zn control” columns) also underwent eight doublings during the same period. These results demonstrate the remarkable ability of the vacuolar store to sustain yeast growth for many generations without available extracellular zinc. Following these periods of growth, total cellular zinc levels, as determined by AAS, reached similarly low levels near the minimum level of zinc required for yeast growth (Fig. 3B).

**Zinc levels and distribution during zinc shock.** Zinc shock occurs when zinc-limited cells are exposed to even modest levels of extracellular zinc. Because zinc-limited cells up-regulate their plasma membrane zinc transporters, newly added zinc can accumulate to very high levels in these cells. Our previous results indicated that the vacuole is required for cells to survive zinc shock (36). To assess the levels and intracellular distributions of zinc during zinc shock, we first used a pulse-chase protocol in conjunction with organelle isolation and AAS/ICP-MS analysis. Cells were zinc limited by growth in CSD medium, their cell walls were removed to form spheroplasts, and they were then treated with 100 μM ZnCl₂. Spheroplasts were used to allow subsequent organelle isolation; levels of zinc accumulation were similar in intact cells and spheroplasts (data not shown). After 10 min, the cells were harvested by centrifugation and analyzed for cellular zinc accumulation by AAS. Alternatively, the zinc-treated cells were resuspended in low-zinc CSD medium and incubated for an additional 10 or 20 min prior to harvesting. Both the wild type and the zrc1Δ cot1Δ mutants accumulated considerable zinc during the 10-min pulse, and this zinc was retained during the 20-min chase period (Fig. 4A). zrc1Δ cot1Δ mutant cells accumulated ~40% less zinc than wild-type cells, presumably because of the lack of vacuolar sequestration.

To examine vacuolar zinc accumulation, the cells were harvested after the initial zinc pulse and after the chase periods and vacuoles were isolated. We were unable to test zrc1Δ cot1Δ mutants in this way because their vacuoles were difficult to isolate following zinc shock due to organelle breakage. No differences in vacuole morphology were observed, and the cause of this vacuolar fragility in zrc1Δ cot1Δ cells is not yet known. Zinc accumulation in wild-type vacuoles was similar to the total zinc accumulation, indicating that most of the zinc accumulated during this treatment was sequestered in the vacuole (Fig. 4B). Finally, mitochondrial zinc levels were examined in zinc-shocked wild-type and zrc1Δ cot1Δ cells (Fig. 4C). Mitochondrial zinc levels in both strains rose rapidly and then decreased during the 20-min chase period. ICP-MS analysis indicated that there was no change in mitochondrial Ca levels during zinc shock, suggesting that the observed changes in zinc were not simply due to different degrees of cation leakage from the mitochondria during their preparation (data not shown). Thus, these data further support the hypothesis that mitochondria have mechanisms to maintain zinc homeostasis.

**Analysis of zinc shock by EPXMA.** EPXMA was also used to examine zinc distribution during zinc shock, and these data are reported in Table 1. Consistent with the AAS/ICP-MS data, total cellular zinc rose to a high level in wild-type cells during zinc shock. Also consistent with these data, wild-type vacuolar zinc increased to a high level. Nonvacuolar cytoplasmic zinc levels remained low relative to vacuolar levels, but an increase in zinc content was detected. This observation confirms that the level of zinc in nonvacuolar compartments does increase during zinc shock.

EPXMA of zrc1Δ cot1Δ mutants during zinc shock also detected large increases in total zinc levels. Surprisingly, rapid vacuolar accumulation of high levels of zinc, albeit to a lower level than that observed for the wild type, was also seen in zrc1Δ cot1Δ mutant cells. This result indicates that, although Zrc1 and Cot1 are important for zinc influx into the vacuole, other transporters also participate in this process under zinc shock conditions. The identity of those other systems is not yet known.

**Effects of zinc status on other elements.** EPXMA allows the simultaneous analysis of several elements in addition to zinc. Therefore, we used this method to determine whether zinc status alters the level and/or distribution of other elements in yeast. The whole-cell levels of Ca, Cl, Fe, K, Mg, and P measured by EPXMA in wild-type cells grown in SD medium are

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**FIG. 4.** Effects of zinc shock on the accumulation and intracellular distribution of zinc. (A) Wild-type (WT) and zrc1Δ cot1Δ mutant cells were grown in zinc-limiting CSD medium overnight to induce zinc deficiency. The cells were then harvested, and their cell walls were removed to generate spheroplasts; this allowed the rapid isolation of organelles assayed as reported below. To induce zinc shock, 100 μM ZnCl₂ was added to the medium for a 10-min period. After this treatment, the cells were washed free of exogenous zinc and analyze...
shown in Fig. 5A. The abundances of P and K were ~10-fold greater than those of Mg and Cl, which in turn were greater than the levels of Ca and Fe. These results are consistent with measurements of these elements in yeast cells as determined by other methods, e.g., ICP-atomic emission spectroscopy (12). Na levels were below the level of detection in cells grown under these conditions. In Fig. 5B to G, the abundances of each element measured in vacuoles and in the nonvacuolar cytoplasmic regions of the cell are plotted. Ca, Mg, and P were found at higher concentrations in the vacuole than in the cytoplasm, while Fe and K were more equally distributed. Cl was more abundant in the nonvacuolar cytoplasm than within vacuoles.

The data reported in Fig. 5 were obtained from cells grown under moderate-zinc conditions (i.e., SD medium with no additional zinc). The effects of zinc status on these various elements under steady-state growth conditions are reported in Table 2. Total, vacuolar, and nonvacuolar cytoplasmic Fe levels were unaffected by changes in zinc status. Ca and Na levels fluctuated in response to zinc, with levels (i) decreased in SD medium-grown cells versus CSD medium-grown cells and (ii) similar in cells grown in CSD medium and cells grown in SD medium plus 100 μM ZnCl₂. Cl levels decreased markedly in all compartments in high zinc. K, Mg, and P all showed statistically significant increased accumulation in response to zinc. For K, this increase was largely due to changes in vacuolar levels and was observed in both SD medium-grown cells and cells grown in SD medium plus 100 μM ZnCl₂ relative to CSD medium-grown cells. In contrast, large increases in both vacuolar and cytoplasmic Mg and P were observed. These increases were seen only in cells grown in SD medium plus 100 μM ZnCl₂, indicating that this effect is a response to high zinc levels.

To determine if the effects of zinc on the accumulation of these elements were rapid or required long-term incubation under specific zinc conditions, we measured the effects of zinc shock on abundance of these elements (Table 2). Consistent with the increase in K with elevated zinc under steady-state conditions, vacuolar K levels increased twofold following 10 min of zinc shock and remained elevated over the 20-min chase period. A statistically significant increase in total cellular K was detected following the 20-min chase period. Thus, the response of K to increasing zinc occurs very rapidly. In contrast, no increase in Mg or P levels was observed over the zinc shock treatment period. Thus, while the effects of zinc on K levels are rapid, the effects of zinc on Mg and P accumulation require longer periods of incubation in high zinc to occur. Zinc shock caused a decrease in vacuolar Ca and a transient rise in cytoplasmic Ca. Following the chase period, cytoplasmic Ca levels returned to the starting level but vacuolar Ca levels were reduced further. Zinc shock also caused a transient decrease in cellular Cl, followed by an increase that occurred during the chase period. This result indicated that the effect of zinc status on Cl observed under steady-state growth conditions also requires a longer period of incubation to occur. Finally, zinc shock was associated with a fivefold increase in vacuolar Na levels whereas total Na levels were not significantly affected. This result suggests a redistribution of cellular Na into the vacuole along with zinc.

EPXMA data were also collected from zrc1Δ cot1Δ mutants grown under steady-state conditions of increasing zinc or following zinc shock. These data are provided in Table S1 in the supplemental material.

The EPXMA data indicated that cellular K, Mg, and P levels rise in response to increased zinc availability under steady-state conditions. To further investigate the correlation between these elements, we plotted those data as scatter plots displaying the results obtained with individual cells. Remarkably, there was a strong positive correlation between Mg and P levels in single cells (Fig. 6). This effect was seen under all treatment conditions and in all compartments (P < 0.0001).
addition, these correlations were observed to occur in \( \text{zrc1} / \text{H9004} \) and \( \text{cot1} / \text{H9004} \) mutant cells, although these mutations did alter the slope of the correlation in whole cells under some conditions \((P < 0.05)\). These results indicate the strong interdependency of intracellular Mg and P in both wild-type and \( \text{zrc1} / \text{H9004} \) and \( \text{cot1} / \text{H9004} \) mutant cells.

To assess the effect of zinc status on the correlation between Mg and P, scatter plots comparing Zn with Mg and Zn with P were prepared (Fig. 7). Figure 7 displays the data for wild-type cells grown in high zinc because this was the only steady-state condition where zinc was detectable by EPXMA. Strong positive correlations between Mg and Zn and between P and Zn were found \((P < 0.0001)\). In contrast, there was no clear correlation between Zn and K either in whole cells or in vacuoles \((P > 0.3)\) (Fig. 7). In addition, no consistent correlation between K and Mg or between K and P was observed (data not shown).

**DISCUSSION**

The yeast vacuole is a major storage site for many metabolites and ions, including amino acids, phosphate, and magnesium (23). A major goal of this study was to determine the level to which zinc can be stored in this compartment. Analyses of purified vacuoles and of vacuoles in situ within cells by EPXMA both demonstrated that remarkably high amounts of zinc are stored in the vacuole under conditions of zinc excess.

Under high-zinc conditions, we found that as much as 900 pmol Zn/10^6 cells could accumulate in the vacuole. Under zinc shock conditions, a similarly high amount of zinc \((i.e., 758 \pm 110 \text{ pmol/10}^6 \text{ cells, as determined by EPXMA})\) accumulated in this compartment. These levels translate into concentrations of almost 100 mM. We suspect that this concentration is near the maximum storage capacity of the organelle, given that yeast cannot tolerate much higher zinc treatment conditions.

In other terms, vacuolar zinc can be as high as \(7 \times 10^8\) atoms per cell. Given that the minimum amount of zinc required for yeast growth is \(~5 \times 10^6\) atoms per cell (34), the vacuolar zinc store can be sufficient to supply this nutrient to many subsequent generations of yeast following transfer from zinc-replete to zinc-limiting media. Given that free-living microbes often face major changes in nutrient availability, the capacities of yeast cells to store zinc and later utilize stored zinc are likely to be of great advantage to these cells growing in the wild. Similarly, vacuolar zinc storage is likely to be an important factor in the successful colonization of host organisms by pathogenic fungi, given that one host response to infection is to withhold zinc from the pathogen (30, 31).

It remains to be determined what factors, aside from the vacuolar zinc transporters themselves, influence the ability of the vacuole to accumulate zinc. One contributing factor is likely to be acidification of that compartment by the vacuolar V-type H^+-ATPase. Vacuolar acidification provides the pro-
phosphate ligands (13). Similarly, stored zinc in mostly vacuolar zinc is bound to a mixture of carboxylate and zinc conditions indicated that intracellular (and therefore high affinity (2, 38). In addition, organic anions, such as glutamate and citrate, accumulate to high levels in the vacuole with high affinity (21). A recent study of other fungal species grown under high-glutamate and citrate, accumulate to high levels in the yeast vacuole (24) and can bind zinc polymer, i.e., long chains of multiple phosphate groups, accumu-
lates to high levels in the yeast vacuole (24) and can bind zinc. Polyphosphate synthesis and found no effect on zinc storage. Over the testable range of zinc levels, our EPXMA studies demonstrate that these and other compartments in the nonvacuolar cytoplasmic region of yeast cells do not serve as major sites of zinc sequestration relative to the vacuole in zinc-treated cells.

Our previous results implicated Zrc1 and Cot1 as the only zinc transporters responsible for zinc uptake into the vacuole (34). These conclusions were supported here in experiments where cells were grown under steady-state conditions; vacuolar zinc accumulation under these conditions was blocked effectively by mutation inactivation of Zrc1 and Cot1. Surpris-
ingly, however, we found that during zinc shock additional pathways for zinc entry into this organelle exist. Following 10 min of zinc shock, while wild-type cells accumulated 18.6 ± 2.4 nmol Zn/mg (dry weight) in the vacuole, zrc1Δ cot1Δ mutants accumulated a lower but still significant amount of vacuolar zinc (6.5 ± 1.3 nmol Zn/mg [dry weight]). These results suggest that additional, albeit less efficient, transporters are present in the vacuolar membrane to mediate zinc import during zinc shock. These transporters may have lower affinity for zinc, and this characteristic would explain why they do not contribute to vacuolar zinc import under steady-state conditions.

A third goal of this study was to determine the importance of vacuole zinc sequestration in buffering the levels of zinc in other compartments. With respect to the mitochondria, we found no such role. Over the testable range of zinc levels, mitochondrial zinc accumulation was unaffected by mutation of the Zrc1 and Cot1 vacuolar zinc transporters. This resistance of the mitochondria to perturbations of cellular zinc homeostasis may be due to the mechanisms in place to maintain mitochondrial zinc homeostasis, as described above. Our ICP-MS results do suggest, however, that the vacuole buffers the zinc levels in other compartments of the cell. When grown in SD medium plus 0 to 25 μM ZnCl2, zrc1Δ cot1Δ mutant cells accumulated as much total zinc as wild-type cells. However, while the wild-type cells put much of that zinc into the vacuole, the zrc1Δ cot1Δ mutant did not. Therefore, excess zinc must

FIG. 7. Correlation between Mg, P, and K contents versus Zn content in individual cells. Vacuole and whole-cell levels of Mg, P, K, and Zn, measured in individual wild-type cells grown in high zinc (SD medium plus 100 μM ZnCl2), are plotted. All values are in nmol/mg (dry weight).
accumulate in some other compartment of these mutant cells, perhaps the cytosol, zinsosomes, or organelles of the secretory pathway.

Finally, we have focused this study mainly on the role of the yeast vacuole as a subcellular storage and distribution site for zinc. It should be noted that the EPXMA and ICP-MS methodologies used measure both free and bound ions/elements; cell homeostasis likely encompasses concomitant interactions of zinc and other elements in both physiological states. That being said, it was intriguing to find that the levels of K, Mg, and P all rise with increased zinc status. In the case of Mg and P, there was a strong correlation between the levels of these elements in individual cells. Phosphorus accumulation is known to affect Mg uptake, and this effect is thought to be due to differences in vacuolar polyphosphate levels (2). However, we found that the correlation between P and Mg also existed in the nonvacuolar cytoplasmic regions of cells. While most polyphosphate accumulates in the vacuole in yeast, some may also accumulate in other compartments (24). Therefore, this extravacuolar pool of polyphosphate may play a role in determining Mg storage elsewhere in the cell. More surprising is the observation that Zn status correlates closely with both P and Mg. Given that P levels appear to determine, at least in part, cellular Mg levels, we suggest that Zn status somehow alters Mg levels indirectly by affecting P levels. How this occurs remains unclear. What is clear is that the effects of Zn on P and Mg accumulation are not determined by the vacuolar pool of zinc; the same effects were also observed in zrc1Δ cot1Δ mutants, in which vacuolar zinc levels are very low. A recent study has shown that polyphosphate accumulation can be affected by many different processes, including ATP generation and primary metabolism (15). We suggest that Zn status affects P through one or more of these indirect mechanisms. Although K levels also increased in response to zinc, several observations indicate that this effect is unrelated to the effects of Zn on Mg and P levels. First, the effects of zinc on K are mostly vacuolar whereas Mg and P are also affected in the cytoplasm. Second, the effects of Zn on K levels were seen during zinc shock while no rapid increase was observed for Mg and P. Finally, no correlation between K and Mg or P in individual cells was observed. Thus, the mechanisms underlying the effects of zinc status on K accumulation are unrelated to those altering (sub) cellular Mg and P levels. These effects and the potentially important role of Na, for example, in these mechanisms need further examination by use of analytical techniques more sensitive than those used here, such as high spatial resolution secondary ion mass spectrometry imaging in combination with molecular-based methods.

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REFERENCES

1. Andreini, C., L. Banci, I. Bertini, and A. Rosato. 2006. Counting the zinc-proteins encoded in the human genome. J. Proteome Res. 5:196–201.
2. Beeler, T., K. Bruce, and T. Dunn. 1997. Regulation of cellular Mg²⁺ by Saccharomyces cerevisiae. Biochem. Biophys. Acta 1323:310–318.
3. Beyermann, D., and H. Haase. 2001. Functions of zinc in signalling, proliferation and differentiation of mammalian cells. Biometals 14:331–341.
4. Cai, L., X. K. Li, Y. Song, and M. G. CHERIAN. 2005. Essentiality, toxicology and chelation therapy of zinc and copper. Curr. Med. Chem. 12:2753–2763.
5. Conklin, D. S., M. R. Cullbertson, and C. Kung. 1994. Interactions between gene products involved in divalent cation transport in Saccharomyces cerevisiae. Mol. Gen. Genet. 244:303–311.
6. Costello, L. C., and R. B. Franklin. 1998. Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer. Prostate 35:285–296.
7. Costello, L. C., Y. Liu, R. B. Franklin, and M. C. Kennedy. 1997. Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. J. Biol. Chem. 272:28875–28881.
8. Desbrosses-Fonrouge, A. G., K. Voigt, A. Schroder, S. Arrivault, S. Thomine, and U. Kramer. 2005. Arabidopsis thaliana MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn co-localisation. FEBS Lett. 579:416–4174.
9. Devirgiliis, C., C. Murgia, G. Danuscher, and G. Perrozzi. 2004. Exchangeable zinc ions transiently accumulate in a vesicular compartment in the yeast Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 323:58–64.
10. Duffy, J. Y., C. M. Miller, G. L. Rutschilling, G. M. Ridder, M. S. Clegg, C. L. Keen, and G. P. Daston. 2001. A decrease in intracellular zinc level precedes the detection of early indicators of apoptosis in HL-60 cells. Apoptosis 6:161–172.
11. Eide, D. J. 2006. Zinc transporters and the cellular trafficking of zinc. Biochim. Biophys. Acta 1763:711–722.
12. Eide, D. J., S. Clark, T. M. Nair, M. Gehl, M. Gribskov, M. L. Gueriniot, and J. F. Harper. 2005. Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in Saccharomyces cerevisiae. Genome Biol. 6:319.
13. Fomina, M., J. Charmock, A. D. Bowen, and G. M. Gadd. 2007. X-ray absorption spectroscopy (XAS) of toxic metal mineral transformations by fungi. Environ. Microbiol. 9:308–321.
14. Frazzini, V., E. Rokach, W. Moccegianini, and S. L. Sensi. 2006. Oxidative stress and brain aging: is zinc the link? Biogerontology 7:307–314.
15. Freimoser, F. M., H. C. Hurlimann, C. A. Jakob, T. P. Werner, and N. Amrhein. 2006. Systematic screening of polyphosphates (poly P) levels in yeast mutant cells reveals strong interdependency with primary metabolism. Genome Biol. 7:R109.
16. Gitan, R. S., H. Luo, J. Rodgers, M. Broderius, and D. Eide. 1998. Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J. Biol. Chem. 273:28267–28274.
17. Glick, B. S., and A. L. Pon. 1995. Isolation of highly purified mitochondria from Saccharomyces cerevisiae. Methods Enzymol. 260:213–223.
18. Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.
19. Ingram, P., J. D. Shelburne, and A. LeFurgey. 1999. Principles and instrumentation, p. 1–58. In P. Ingram, J. D. Shrubler, V. Roggli, and A. LeFurgey (ed.), Biomedical applications of microprobe analysis. Academic Press, San Diego, CA.
20. Kamizono, A., M. Nishizawa, Y. Teranishi, K. Murata, and A. Kimura. 1989. Identification of a gene conferring resistance to zinc and cadmium ions in the yeast Saccharomyces cerevisiae. Mol. Gen. Genet. 219:161–167.
21. Kitamoto, K., K. Yoshizawa, Y. Ohashi, and Y. Anraku. 1988. Dynamic aspects of vacuolar and cytosolic amino acid pool of Saccharomyces cerevisiae. J. Bacteriol. 170:2683–2686.
22. Kleineke, J. W., and I. A. Brand. 1997. Rapid changes in intracellular Zn⁺⁺ in rat hepatocytes. J. Pharmacol. Toxicol. Methods 38:181–187.
23. Klionsky, D. J., P. K. Kang, and S. R. Emr. 2001. The fungal vacuole: composition, function and biogenesis. Microbiol. Rev. 54:266–292.
24. Kornberg, A., N. N. Rao, and D. Auli-Riche. 1999. Inorganic polyphosphate: a molecule of many functions. Annu. Rev. Biochem. 68:93–125.
25. LeFurgey, A., S. Davilla, D. A. Kopf, J. R. Sommer, and P. Ingram. 1992. Real-time quantitative elemental analysis and mapping: microchemical imaging in cell physiology. J. Microsc. 165:191–223.
26. LeFurgey, A., M. Gannon, J. Blum, and P. Ingram. 2005. Leishmania donovani amastigotes mobilize organic and inorganic osmoles during regulatory volume decrease. J. Eukaryot. Microbiol. 52:277–289.
27. LeFurgey, A., P. Ingram, and J. J. Blum. 2001. Compartmental responses to acute osmotic stress in Leishmania major result in rapid loss of Na⁺ and Cl⁻. J. Comp. Physiol. B 171:385–394.
28. LeFurgey, A., P. Ingram, and J. J. Blum. 1990. Elemental composition of polyphosphate-containing vacuoles and cytoplasm of Leishmania major. Mol. Biochem. Parasitol. 40:77–86.
29. Li, L., S. Chen, D. McVey Ward, and J. Kaplan. 2001. CCC1 is a transporter that mediates vacuolar iron storage in yeast. J. Biol. Chem. 276:29515–29519.
30. Linzzi, J. P., I. A. Lichten, S. Rivera, R. K. Blanchard, T. B. Aiyemidim, M. D. Knauf, T. Ganz, and R. J. Cousins. 2005. Interleukin-1 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc. Natl. Acad. Sci. USA 102:6843–6848.
31. Liljof, S. J., B. L. Hahn, and P. G. Sohle. 2004. Fungal susceptibility to zinc deprivation. J. Lab. Clin. Med. 144:208–214.
32. Lyons, T. J., A. P. Gasch, L. A. Gaither, D. Botstein, P. O. Brown, and D. J. Eide. 2000. Genome-wide characterization of the Zap1 zinc-responsive regulon in yeast. Proc. Natl. Acad. Sci. USA 97:7957–7962.
33. Ma, J. F., D. Ueno, F. J. Zhao, and S. P. McGrath. 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of Thlaspi caerulescens. Planta 220:731–736.

34. MacDiarmid, C. W., L. A. Gaither, and D. Eide. 2000. Zinc transporters that regulate vacuolar zinc storage in Saccharomyces cerevisiae. EMBO J. 19: 2845–2855.

35. MacDiarmid, C. W., M. A. Milanick, and D. J. Eide. 2002. Biochemical properties of vacuolar zinc transport systems of Saccharomyces cerevisiae. J. Biol. Chem. 277:39187–39194.

36. MacDiarmid, C. W., M. A. Milanick, and D. J. Eide. 2003. Induction of the ZRC1 metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J. Biol. Chem. 278:15065–15072.

37. Miyabe, S., S. Izawa, and Y. Inoue. 2001. Zrc1 is involved in zinc transport system between vacuole and cytosol in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 282:79–83.

38. Moe, O. A., and S. A. Wiest. 1977. Determination of stability constants for zinc-pyrophosphate complexes. Anal. Biochem. 71:73–78.

39. Munujos, P., J. Coll-Canti, F. Gonzalez-Sastre, and F. J. Gella. 1993. Assay of succinate dehydrogenase activity by a colorimetric-continuous method using iodonitrotetrazolium chloride as electron acceptor. Anal. Biochem. 212:506–509.

40. Outten, C. E., and T. V. O’Halloran. 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492.

41. Palminteri, R. D., T. B. Cole, and S. D. Findley. 1996. Zrt-T-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. EMBO J. 15:1784–1791.

42. Roberts, C. J., C. K. Raymond, C. T. Yamashiro, and T. H. Stevens. 1991. Methods for studying the yeast vacuole. Methods Enzymol. 194:644–661.

43. Sarret, G., P. Saumitou-Laprade, V. Bert, O. Proux, J. L. Hazemann, A. Traverse, M. A. Marcus, and A. Mancheau. 2002. Forms of zinc accumulated in the hyperaccumulator Arabidopsis halleri. Plant Physiol. 130:1815–1826.

44. Sensi, S. L., D. Ton-That, P. G. Sullivan, E. A. Jonas, K. R. Gee, L. K. Kaczmarek, and J. H. Weiss. 2003. Modulation of mitochondrial function by endogenous Zn\(^{2+}\) pools. Proc. Natl. Acad. Sci. USA 100:6157–6162.

45. Sensi, S. L., D. Ton-That, J. H. Weiss, A. Rothe, and K. R. Gee. 2003. A new mitochondrial fluorescent zinc sensor. Cell Calcium 34:281–284.

46. Warley, A. 1997. X-ray microanalysis for biologists, p. 227–229. Portland Press, London, United Kingdom.

47. Waters, B. M., and D. J. Eide. 2002. Combinatorial control of yeast FET4 gene expression in response to iron, zinc, and oxygen. J. Biol. Chem. 277:33749–33757.

48. White, C., and G. M. Gadd. 1987. The uptake and cellular distribution of zinc in Saccharomyces cerevisiae. J. Gen. Microbiol. 133:727–737.

49. Yaiffe, M. P. 1991. Analysis of mitochondrial function and assembly. Methods Enzymol. 194:627–643.

50. Zhao, H., and D. Eide. 1996. The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc. Natl. Acad. Sci. USA 93:2454–2458.

51. Zhao, H., and D. Eide. 1996. The ZRT2 gene encodes the low affinity zinc transporter in Saccharomyces cerevisiae. J. Biol. Chem. 271:23203–23210.

52. Zhao, H., and D. J. Eide. 1997. Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in Saccharomyces cerevisiae. Mol. Cell. Biol. 17:5044–5052.