Metal Ion Uptake in Eukaryotes

Research on *Saccharomyces cerevisiae* reveals complexity and insights about other species

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Copper, iron, manganese, zinc, and other d-block transition elements are essential nutrients that are critical to health. More people in developed countries now suffer from malnutrition due to deficiencies of micronutrients such as metal ions than from protein or energy deficiency. Furthermore, several human diseases, including hemochromatosis, acrodermatitis enteropathica, and Menkes disease, result from mutations that alter metal ion metabolism. In agriculture, metal ions in soil play a major role in determining crop yields. Metal ions are also essential for microbial growth, sometimes serving as important determinants of pathogenicity and other times enabling free-living microbes to compete with other organisms for limited environmental resources. Overaccumulation of metal ions can be toxic. When the intracellular level of a metal ion rises to some critical level, the metal can interfere with vital processes and result in cell death. For example, iron and copper are potent generators of reactive oxygen species that can damage lipids, proteins, and DNA. Because metal ions are both essential and potentially toxic, intracellular metal ion concentrations are subject to precise homeostatic regulation.

Metal ions are required for an amazing variety of biochemical processes. Iron, for example, readily donates and accepts electrons from substrates and can display a broad range of oxidation-reduction potentials depending on the ligand environment surrounding the metal ion. Hence, iron is an important cofactor of several redox-active metalloenzymes such as ribonucleotide reductase and succinate dehydrogenase. Moreover, iron is required for heme biosynthesis and for the activity of many heme-containing proteins such as catalase, the cytochromes of electron transport chains, and hemoglobin.

With the exception of lactobacilli, which use cobalt and manganese as biocatalysts in place of iron, all microorganisms that have been examined require iron.

Zinc, which has only one biologically relevant valence (+2), is essential because it is a catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, carbonic anhydrase, and several carboxypeptidases. Zinc also plays a critical structural role in enzymes and many noncatalytic proteins. For example, several motifs found in transcriptional regulatory proteins are stabilized by zinc, including the zinc finger, zinc cluster, RING finger, and LIM domains. Proteins containing these domains are very common, with as many as 1% of all human gene products containing zinc finger domains. Moreover, according to analysis of the *Saccharomyces* genome, more than 2% of all yeast proteins, more than 150 of 6,000 total gene products, contain zinc-binding domains.

Until recently, little was known about how eukaryotic cells take up metal ions or how they regulate accumulation in response to ion availability and metabolic demand. Over the last five years, however, researchers studying the baker’s yeast *Saccharomyces cerevisiae* identified several genes involved in these processes by means of genetic and molecular techniques. Moreover, yeast genes have been identified because of their similarity to metal metabolism genes of other organisms.

Now this yeast is becoming a useful model system for understanding metal ion uptake and regulation in other eukaryotic cells. For example, studies of *S. cerevisiae* led researchers to identify genes from other eukaryotes that play similar roles in metal ion uptake. Yeast studies are also providing valuable insights into a long-recog-
nized interaction between copper and iron metabolism in mammals.

**Metal Ion Uptake in *S. cerevisiae* Is Tightly Regulated**

Studies conducted in the 1950s, 1960s, and 1970s suggested that a single “divalent cation transport system” mediates uptake of Cd, Co, Mn, Ni, and Zn, and possibly Cu and Fe, which were not examined in those studies. Although widely accepted, this hypothesis of a single, multisubstrate metal uptake system raises many questions. How could such a system maintain an adequate supply of each metal while avoiding any toxic consequences? For example, if Zn was present in limiting amounts but Mn levels were high, how could a single system control the intracellular levels of these two metal ions?

Recent studies indicate that metal ion uptake in *S. cerevisiae* is remarkably more complex than this early picture portrays it. We now know that baker’s yeast has multiple, substrate-specific systems to take up each metal ion (Figure 1). For Cu, Fe, and Zn, separate high-affinity systems are responsible for providing each of these metal ions to the cell when it is in short supply. These systems are tightly regulated, with any one of them increasing activity by more than 10-fold when its metal becomes limited in a cell. Metal-responsive regulatory proteins control this process, activating transcription of the transporter genes when the intracellular concentrations of their respective substrates are low. (A high-affinity Mn-specific uptake system has also been identified, but its regulation has not yet been described.) In addition to these high-affinity systems, low-affinity systems play a “housekeeping” role, supplying each of these metal ions when they are more abundant in the environment. Some low-affinity systems are also metal regulated, but to a far lesser extent than their high-affinity cousins.

**Iron Uptake Is a Two-Step Process in Yeast**

Uptake of iron in *S. cerevisiae* is a two-step process. Extracellular Fe(III) is first reduced to Fe(II) by plasma membrane Fe(III) reductases that are the products of the *FRE1* and *FRE2* genes. These gene products resemble the gp91 phox subunit of cytochrome b<sub>558</sub>, the phagocyte respiratory burst oxidoreductase. Subsequently, Fe(II) enters the cell by one of two different systems. A low-affinity system plays this role in iron-replete cells, with the *FET4* gene encoding the key transport protein.

When iron becomes limiting, a second uptake system is induced that has a 200-fold higher affinity for iron [apparent *K*<sub>m</sub> = 0.15 μM total Fe(II) or 3 nM free Fe(II)]. This high-affinity system relies on two genes, *FET3* and *FTR1*. *FET3* encodes a member of the multicopper oxidase family, which includes ascorbate oxidase, laccase, and ceruloplasmin. Unlike these secreted proteins, Fet3p is an integral membrane protein with a single transmembrane domain. The *FTR1* gene, which apparently encodes the transport protein of this high-affinity system, has six potential transmembrane domains and several potential metal-binding domains.

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**Figure 1**

The known transport systems for metal ions in *S. cerevisiae*. 
How do Fet3p and Ftr1p work? According to one model, the two proteins form a complex in which Fet3p oxidizes the Fe(II) produced at the plasma membrane to Fe(III), which Ftr1p transports across the plasma membrane into the cytoplasm (Figure 2). But why are opposing enzymatic reactions, Fe(III) reduction and Fe(II) oxidation, occurring on the cell surface? One possible answer is that this oxidase-permease mechanism provides substrate specificity to the process through the sequential recognition of Fe(II) by Fet3p and of Fe(III) by Ftr1p. If so, why can't cells simply accumulate the Fe(III) already present in the medium? The most likely answer to this question lies in the solution chemistry of iron. Most soluble Fe(III) is bound to chelators and may not be suitable substrates for uptake. The high-affinity Fe(II) system in S. cerevisiae is tightly regulated at the transcriptional level in response to iron availability. Iron-limited growth increases the steady-state levels of mRNA derived from FRE1, FRE2, FET3, FTR1, and CCC2. Sequences that are required for this increase have been identified in the promoters of these genes. The transcriptional activator protein encoded by the AFT1 gene binds specifically to these sequences, and iron inhibits this binding. Aft1p may itself be an iron-binding protein whose activity (e.g., DNA binding affinity, nuclear localization, etc.) is directly altered by this interaction.

**The Copper-Iron Connection in Yeast and Mammals**

The importance of a multicopper oxidase, Fet3p, in iron uptake suggests a close link between copper metabolism and iron uptake in *S. cerevisiae*. Indeed, copper-deficient cells have low levels of iron uptake activity, and that activity decreases in mutant cells defective for copper uptake (i.e., *crr1* mutants). Adding copper to the medium suppresses the iron uptake defect of these mutants but does not restore copper uptake.

A similar connection between copper and iron transport occurs in mammals. The multicopper oxidase ceruloplasmin may promote transport of iron across mammalian cell membranes. Ceruloplasmin, a serum protein that can oxidize Fe(II) to Fe(III), is present only in low levels in copper-deficient animals, which cannot export iron from the intestinal mucosal cells into the portal bloodstream. Blood iron levels rise rapidly when either copper or ceruloplasmin is injected into these animals. In humans, a mutation in the ceruloplasmin gene blocks synthesis of this protein. This defect wreaks havoc with iron metabolism, leading to low serum iron levels and anemia. Thus, the multicopper oxidase activities of both ceruloplasmin and Fet3p are needed to move iron across cellular membranes. How these oxidases mobilize iron in opposite directions, with Fet3p transporting iron into cells and ceruloplasmin exporting it, is not understood. Perhaps Fet3p is coupled to an Fe(III) transporter, while ceruloplasmin works in conjunction with an Fe(II) transporter, oxidizing the iron to Fe(III) as it exits the cell.

Analysis of the yeast CCC2 gene reveals another exciting parallel between copper and iron metabolism in yeast and mammals. CCC2 encodes a P-type ATPase transporter that is remarkably similar to the proteins encoded by the Menkes syndrome (MNK) and Wilson disease (WD) genes in humans. The CCC2 protein delivers copper to Fet3p in a post-Golgi compartment of the cellular secretory system. While
ecco2 mutants have no obvious defects in plasma membrane copper uptake or intracellular levels of copper, high-affinity Fe(II) uptake is diminished. Both MNK and WD apparently encode intracellular copper transporters that pass the metal ion from the cytoplasm into an otherwise uncharacterized secretory organelle. In the human liver, the WD protein transports copper, which is excreted in the bile or loaded into ceruloplasmin and then secreted into the blood plasma. The MNK protein exports copper from other tissues, including the intestinal mucosa. Thus, mutations in the MNK gene block absorption of dietary copper. Whether a similar copper-iron connection exists in plants is not known. However, starving plants for copper stimulates Fe(III) reductase activity, suggesting that copper deficiency prevents plants from transporting iron and leads to iron deficiency. The recent identification of a putative iron transporter gene from Arabidopsis may allow us to address whether copper is indeed required for iron transport in plants.

**Yeast Copper Uptake Also Has Dual Systems**

As with iron uptake, separate high- and low-affinity systems mediate copper uptake in *S. cerevisiae*. Moreover, in the high-affinity copper uptake system, plasma membrane enzymes first reduce Cu(II) to Cu(I). In fact, the product of the FRE1 gene, a reductase involved in iron uptake, is largely responsible for producing Cu(I), which the high-affinity transporter protein encoded by the CTR1 gene brings into the cell. The low-affinity transporter is not yet characterized.

Cu(II) reductase activity and high-affinity copper uptake are also regulated at the transcriptional level by the product of the MAC1 gene. Mac1p is a metal-responsive transcriptional activator that controls the expression of CTR1 and FRE1 in response to copper status. Copper-limited cells express these genes at high levels, whereas copper-replete cells show only low-level expression. Mac1p has sequence similarity to two other copper-responsive transcriptional activators, Ace1p and Amt1p.

**Manganese Uptake Has Complex Genetics**

* S. cerevisiae also has at least two manganese uptake systems. The apparent *Km* of the high-affinity system is 0.3 μM, and the apparent *Km* of the low-affinity system is approximately 60 μM. The SMF1 gene may encode the 575-amino-acid transporter protein of the high-affinity system, which contains 10 potential transmembrane domains. The product of the SMF2 gene in *S. cerevisiae* is closely related to Smt1p and may encode the low-affinity manganese transporter. When overexpressed, the SMF1 gene suppresses a temperature-sensitive mutation in the MIF1 (MAS1) gene, which encodes a subunit of a protease that acts specifically on mitochondrial protein precursors. Why are MIF1 and SMF1 linked? The first clue to this puzzle is that the in vitro activity of the MIF1 protease is dependent on Mn. Another clue comes from an unusual allele of the CDC1 gene, which is required for intrachromosomal recombination and nuclear division. High levels of manganese in the medium or overexpression of the SMF1 gene suppress this allele.

Sequences in these putative manganese uptake proteins of yeast are more than 50% similar to members of the natural resistance-associated macrophage protein or “Nram” family, which are found in an array of eukaryotes, including mice, humans, and birds as well as plants, insects, and nematodes; they are also found in bacteria. The role of SMF1 in *S. cerevisiae* suggests that these genes may be involved in metal ion transport. Some members of this family of genes are of great medical importance. In mice, the Nramp protein encoded by the *Bcg* gene is required for resistance to a variety of intracellular pathogenic organisms, such as *Mycobacterium bovis*, *Salmonella typhimurium*, and *Leishmania donovani*. During the early immediate phase of infection, macrophage cells engulf such pathogens and sequester them within the phagosome, where they are destroyed by reactive oxygen and nitrogen compounds. However, this ability to kill intracellular pathogens is severely compromised in *bcg* mutant mice.

Some investigators speculate that Nramp proteins act as nitrate/nitrite transporters. On the basis of the role of SMF1 in *S. cerevisiae*, an alternative is that they facilitate pathogen killing by robbing bacteria of manganese, which is re-
quired for superoxide dismutase (SOD), an enzyme that helps bacteria resist the phagocyte oxidative burst. A third alternative, which we favor, is that the bsg mutant macrophages generate little or no reactive oxygen and nitrogen compounds because of a deficiency of manganese or some other metal ion.

**Using Yeast To Identify Metal Transporter Genes from More Complex Eukaryotes**

*S. cerevisiae* mutants defective for metal ion uptake help in identifying and isolating similar genes in other eukaryotes. One proven method, functional expression cloning, involves constructing a plasmid library of cDNAs from an organism of interest and testing those genes in an appropriate mutant yeast strain. This method offers the advantage of isolating genes based on function rather than sequence similarities.

Karlheinz Kampfenkel, of the Universität Potsdam, Germany, was the first to successfully apply this technique to metal ion uptake, using the yeast copper uptake mutant *ctr1* and a gene library from the plant *Arabidopsis thaliana*. The *Arabidopsis* *COPT1* gene suppresses the growth defect of the *ctr1* strain on a copper-limiting medium. Although the plant *COPT1* and the yeast *CTR1* proteins are not alike, the *COPT1* protein apparently is a copper transporter. This 169-amino-acid protein is highly hydrophobic with three potential transmembrane domains, and its amino-terminal 44 amino acids contain a methionine-rich putative metal binding domain similar to those found in Ctr1p and a bacterial copper ATPase transporter protein, the CopB protein of *Enterococcus hirae*. *COPT1* is expressed in flowers, stems, and leaves, but expression has not been detected in roots.

Based on *COPT1*, a similar gene, designated *CTR2*, was identified in the *S. cerevisiae* genome. However, a mutation in *CTR2* does not alter copper requirements, even in a strain in which the *CTR1* gene is also disrupted. Thus, *CTR2* probably does not encode the low-affinity copper transporter in yeast. Nonetheless, the *ctr2* mutant is more resistant to copper in the medium, suggesting that this gene plays some role in copper homeostasis. Perhaps *CTR2* is involved in the intracellular compartmentalization of copper.

**Identifying the ZRT/IRT-Related Protein (ZIP) Family of Metal Ion Transporters**

Iron is an essential nutrient for plants. Approximately one-third of the world’s soils are considered to be iron-deficient. Even when abundant in soil, iron is all but unavailable to plants because the oxidized form, Fe(III), is extremely insoluble. Many plant species have efficient systems, designated strategy I and strategy II, for obtaining iron from their environment. Strategy II plants, which include all grasses, release Fe(III)-binding compounds called phytosiderophores from their roots to bind iron and be absorbed as a complex. Most other plants use strategy I, which is very similar to the iron uptake mechanism used by *S. cerevisiae*; Fe(III) is reduced to Fe(II) by plasma
membrane reductases prior to its uptake by Fe(II)-specific transporters.

Using the functional expression cloning method, we isolated an iron transport gene from the strategy I plant Arabidopsis. Expression of the Arabidopsis IRT1 (for iron-regulated transporter) gene in yeast restores iron-limited growth to a fet3 fet4 double mutant. As anticipated, IRT1 enables yeast cells to take up Fe(II) rather than Fe(III), and other metal ions such as Cu(I), Cu(II), Mn(II), and Zn(II) do not inhibit this process. The predicted protein product of IRT1 contains 339 amino acids and eight potential transmembrane domains. Because IRT1 is expressed in Arabidopsis roots and is induced by iron-deficient growth conditions, we believe that this protein acts as a root Fe(II) transporter.

Because IRT1 is the first iron transporter gene isolated from plants, it will likely prove useful for studying iron uptake in other plants. Moreover, IRT1 led us to discover a second group of transporters involved in metal ion uptake (Figure 3), which we have named the “ZIP” gene family (for ZRT/IRT-related proteins). Two members of this family, ZRT1 and ZRT2, encode zinc transporters in S. cerevisiae (see below). At this time, at least 13 ZIP family members have been identified, including seven plant genes (six from Arabidopsis and one from rice), two genes in S. cerevisiae, one gene in nematodes, one in mice, and two in humans. Based on our studies of IRT1 and the ZRT genes, we propose that other genes in this family may also function as metal ion transporters.

Identifying Zinc Transporters in S. cerevisiae

Finding the IRT1 homologs ZRT1 and ZRT2 in S. cerevisiae prompted us to examine their roles in metal accumulation. A zrt1 mutant cannot grow in 1 mM EDTA, whereas the wild type can. Only zinc restores growth of the mutant strain, suggesting that ZRT1 is required for obtaining zinc.

We already knew that zinc uptake in S. cerevisiae is transporter mediated by at least two systems, one with high affinity [apparent $K_m = 1 \mu M$ total Zn(II), 10 nM free Zn(II)] and a second with lower affinity [apparent $K_m = 10 \mu M$ total Zn(II), 100 nM free Zn(II)]. The ZRT1 gene appears to encode the transporter protein of the high-affinity system, whereas the ZRT2 gene encodes the transporter of the low-affinity system. Additional zinc uptake systems are also present in S. cerevisiae. Because the zrt1 zrt2 double mutant is viable, an additional but unidentified system can provide yeast with zinc.

In response to zinc-limiting growth conditions, the activity of the high-affinity system is induced 100-fold, with control being at the transcriptional level. Despite the 75-fold-higher extracellular zinc level required to down-regulate this promoter in zrt1 mutant cells, the ZRT1-lacZ fusion gene responds similarly to intracellular zinc levels in both the wild type and zrt1 mutants. We hypothesize that there is a zinc-responsive transcriptional regulator that resembles the Mac1p protein that regulates copper uptake.

Research on Metal Ion Metabolism Is Changing

Research on metal ion uptake in S. cerevisiae has provided a wealth of reagents and information to drive progress in this field. Information about yeast genes involved in accumulating metal ions, including those that encode transporter, regulatory, and accessory proteins such as reductases and oxidases is already having a major impact on the study of metal ion uptake in other organisms, including plants and humans. With these yeast genes in hand, we can more readily scrutinize the Nramp and ZIP family proteins. Until the structures of these membrane bound transporters are determined by crystallography, we will be relying heavily on mutational analysis. The yeast genes and proteins also are leading to a more detailed analysis of how transcriptional and posttranscriptional mechanisms regulate metal uptake and how intracellular metal ion concentrations are controlled.

Studies of such genes will influence other areas of metal ion research. For example, they provide a means to manipulate intracellular metal ion concentrations when studying metal toxicity. Iron overaccumulation in yeast leads to double-strand DNA breaks, according to Caroline Philpott and Richard Klausner of the National Cancer Institute in Bethesda, Md. For some microbial pathogens, virulence depends on the microorganism’s ability to obtain metal
ions from its host. Agents that interfere with metal uptake may provide new antibiotics against pathogens.

For example, certain compounds that resemble but interfere with the iron-scavenging siderophores of bacteria and fungi can kill such cells. Studies of the yeast metal ion uptake systems have deepened our understanding of the underlying causes of several human genetic diseases. Finding the protein in *S. cerevisiae* that is similar to those encoded by the Wilson disease and Menkes syndrome genes helped confirmed their role in intracellular copper transport. Perhaps the greatest potential for practical applications is in agriculture. By making plants more efficient at obtaining metal ions from soils, we could accelerate plant growth and also improve their nutritional value. Furthermore, such plants may be useful in removing toxic heavy metals from contaminated sites. For instance, Richard Meagher of the University of Georgia, Athens, Ga., has constructed transgenic plants that express a bacterial *merA* gene, enabling them to sequester soil mercury by converting Hg(II) to Hg(0). Biased rhizospheres, in which both the microbial and plant species are designed to mobilize metals from soils, is another exciting area of research.

SUGGESTED READING


