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Abstract:
Transition metal ions are required for many aspects of mitochondrial physiology. Copper, iron, manganese and zinc are cofactors in metalloenzymes and metalloproteins within the organelle. Little is known how cells maintain optimal pools of these metal ions for mitochondrial function. This review documents the available literature on mitochondrial metal ion pools and protein metallation reactions. Upon perturbation in metal pools, mis-metallation reactions do occur. Thus, regulation of metal ion accessibility and bioavailability must exist.

Mitochondrial Structure

The mitochondrion consists of a continuous tubular reticulum that makes up nearly 10% of the cell volume in respiring yeast cells. The tubular reticulum is

highly dynamic undergoing continuous fusion and fission events that are essential to preserving mitochondrial structure and function (Hermann and Shaw 1998). The organelle is enclosed by a double membrane (Frey and Mannella 2000). An aqueous volume between the two membranes is called the intermembrane space (IMS) and is interrupted by junction points in which the inner membrane (IM) and outer membrane (OM) are in contact. The volume enclosed within the IM is the matrix compartment. The IM invaginates in cells with high respiration rates creating tubular entities designated cristae. Cristae are enriched in the five enzyme complexes involved in oxidative phosphorylation (Tzagoloff 1982). The respiratory enzyme complexes include the NADH oxidase (complex I), succinate dehydrogenase (complex II), cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase (complex V).

Of the myriad of functions of the mitochondrion, one significant role is the generation of ATP through respiratory reduction of oxygen to water and oxidation of NADH and FADH2. Proton pumping across the IM arising from electron transfer reactions in the reduction of O_2 drives ATP synthesis. Fatty acid oxidation is a key metabolic process localized within mitochondria. One paricular lipid in the mitochondrial IM is the diphosphatidylglycerol called cardiolipin (Zhang et al. 2002). This lipid is synthesized within the mitochondrion and is important in the structural integrity of the IM and activities of the respiratory chain (Zhang et al. 2005a).

Yeast mitochondria are also responsible for FeS cluster biogenesis and heme formation. Heme transport from mitochondria is necessary for extramitochondrial hemoproteins. In contrast, cytosolic and nuclear Fe/S proteins require a product of the mitochondrial FeS cluster biosynthetic pathway that is matured to Fe/S clusters within the cytoplasm by a distinct protein machinery (Lill et al. 1999; Lill and Muhlenhoff 2006). Since FeS is a necessary cofactor to the Rli1 protein involved in ribosome biogenesis, FeS biogenesis is one essential function of mitochondria (Kispal et al. 2005).

Metal ions usage within Mitochondria

Iron is present in mitochondrial FeS centers and hemoproteins. FeS centers are present in the matrix proteins biotin synthase, aconitase, homoaconitase and ferredoxin. In addition, complex I of the respiratory chain contains 9 distinct Fe/S centers. Complexes II-IV of the respiratory chain require heme cofactors. The soluble hemoprotein, flavohemoglobin, exists in the yeast mitochondrial matrix as well as the cytoplasm (Cassanova et al. 2004). Cytochrome *c* peroxidase is a heme-containing enzyme within the IMS. Iron is also an important cofactor in a class of enzymes using oxo-bridged binuclear iron centers. The ferroxidase center in mitochondrial ferritin contains a diiron carboxylate center (Levi et al. 2001). A related site is present in the alternative oxidase (AOX) found in the mitochondrial respiratory chain of plants and some fungi (Maxwell et al. 1999).

Zinc is equally abundant to iron within mitochondria in yeast cells cultured in rich medium. Within the mitochondria, zinc is a cofactor of Zim17, a protein that interacts with Ssc1 contributing to both protein import and protein folding within the matrix (Burri et al. 2004). Zinc is likely a cofactor for Mdj1 that is the DnaJ component of the Ssc1 chaperone system for folding of newly imported mitochondrial proteins within the matrix. A significant fraction of the cellular zinc is associated with ribosomal proteins in the cytoplasm. These proteins have a conserved duplicated CxxC sequence motif with the thiolates serving as ligands for Zn(II). A subunit of the mitochondrial ribosome is MRPL32 that has a duplicated CxxC motif and therefore is a likely Zn-binding subunit. Zn-metalloenzymes are involved in several mitochondrial metabolic functions including alcohol oxidation (Adh3, Adh4) and leucine biosynthesis (Leu9). A series of mitochondrial metalloproteinases including Yme1, Pdr1 and the Yta10/Yta12 AAA proteinase are Zn-metalloenzymes. The Cox4 subunit of cytochrome *c* oxidase (CcO) is a Zn-requiring molecule with Zn(II) binding contributing to the stability of Cox4. Zinc also appears important in the protein import mediated by small Tim proteins (Tim9/10) in the TIM22 import pathway

(Koehler 2004). Protein import into the IMS through the Mia40 pathway may also be facilitated by Zn(II) binding to maintain the thiolates in proteins in the import competent state (Lu and Woodburn 2005; Rissler et al. 2005).

Copper is only about 20% of iron and zinc levels in yeast mitochondria. Within the mitochondrion, copper is required for assembly of CcO and superoxide dismutase (Sod1) (Sturtz et al. 2001; Tsukihara et al. 1995). Two mitochondrially-encoded subunits of CcO have copper centers. A series of low abundance proteins (Cox17, Cox19, Sco1, Cox11) within the mitochondrial IMS contribute to the copper metallation of CcO during its biogenesis and these proteins transiently bind copper ions (Carr and Winge 2003). In the absence of copper metallation of CcO, the enzyme complex is unstable and degraded. Approximately 1-5% of total cellular Cu,ZnSod1 is localized within the IMS and its localization within this compartment is dependent on Cu-binding Ccs1 (Sturtz et al. 2001).

The only known function of Mn(II) in mitochondria is as a cofactor in MnSod2 (Luk and Culotta 2001). MnSod2 protects against oxidative damage by scavenging superoxide anions. It is imported into the matrix as an apo-protein and the metallation is probably coupled to the folding reaction because Mn(II) cannot be loaded into a folded apo-Sod2 (Luk et al. 2005).

Metal ion transport in mitochondria

Ions are transported across the mitochondrial membranes either by channels or translocases. The OM is porous by virtue of the Tom40 and porin channels. Translocation of unfolded preproteins occurs through the Tom40 channel. Porin channels permit diffusion of ions and molecules less than 1 kDa. The IM is impermeable to most ions, and thus ion transport requires permeases. Protein import across the IM is mediated by the TIM complex and the import process is ATP-dependent and requires a membrane potential. The channel formed by Tim23 is gated by Tim50 ensuring maintenance of the proton gradient across the IM (Meinecke et al. 2006).

Transport of metabolites into the matrix such as pyruvate, citrate, fatty acids in the form of acylcarnitine derivatives, amino acids and ADP/P is facilitated by translocases. Many of these translocases are members of the mitochondrial carrier family (MCF) of proteins consisting of tandem repeats of a helix-loop-helix motif (Robinson and Kunji 2006). Two of the 35 yeast MCFs are metal ion transporters. Mrs3 and Mrs4 are redundant iron transporters. Cells lacking these two transporters have reduced mitochondrial iron pools (Foury and Roganti 2002). The form of iron transported by Mrs3/Mrs4 is unclear. In a computational study of MCF proteins based on the structure of the ADP/ATP carrier MCF, Robinson and Kunji identified key contact residues in MCFs that appear to recognize and distinguish substrates. Based on these analyses, Mrs3/4 are predicted to transport a complex distinct from a keto or amino acid (Robinson and Kunji 2006). Two other mitochondrial IM proteins (Mmt1 and Mmt2) are iron transporters (Li and Kaplan 1997). Depletion of Mmt1 and Mmt2 in yeast confers a growth defect in low iron medium. No transporters for Zn(II), Mn(II) and Cu(I)/Cu(II) have been identified.

Protein metallation within the mitochondrion

Mitochondrial metalloproteins are folded within the IMS or the matrix, so metal ion insertion occurs within these compartments. The pathway of metal ion insertion into metalloproteins during the folding reaction is a topic of intense study (Luk et al. 2003b). The only well studied pathway of metal ion insertion in eukaryotic cells is the metallochaperone-mediated pathway in the cytoplasm. Metallochaperones mediate copper ion shuttling to sites of copper utilization (Huffman and O'Halloran 2001). Protein-mediated copper targeting imparts metal ion specificity to transfer reaction. The Atx1 metallochaperone shuttles Cu(I) to the Ccc2 P-type ATPase transporter localized in post-Golgi vesicles (Lin et al. 1997). Ccs1 provides Cu(I) for activation of Sod1 (O'Halloran and Culotta 2000). Ccs1 acts on newly synthesized Sod1 molecules to insert Cu(I) and in addition to catalyze formation of an essential disulfide bond in Sod1 (Furukawa et al. 2004).

A Ccs1-bypass mechanism for Cu-metallation of Sod1 exists in mammalian cells as partially active Sod1 is present in mice lacking Ccs1 (Wong et al. 2000). Cu(I) delivery from Ccs1 and Atx1 to target proteins occurs via ligand exchange reactions within heterodimeric complexes (O'Halloran and Culotta 2000). The requirement for specific protein:protein interaction imparts selectivity in metal transfer reactions. Thus, Sod1 cannot be copper metallated by Atx1 and Ccc2 cannot receive Cu(I) through Ccs1. Metallochaperones have not been identified for zinc, iron and manganese, so the pathway of metal ion insertion with these metals remains unresolved.

The metallochaperone paradigm predicts that copper metallation reactions within the mitochondrion may also be mediated by shuttle proteins that imparts specificity to the transfer reactions. Within the IMS, Cox17 and Ccs1, serve this function in the Cu(I) metallation of CcO and Sod1, respectively. The function of Cox17 in CcO metallation occurs through two IM accessory proteins that impart specificity in the transfer to the two distinct copper centers in CcO. Sco1 and Cox11, appear to function as co-metallochaperones in the assembly of the CuA and CuB sites in CcO (Carr and Winge 2003).

A fraction of Sod1 exists within the mitochondrial IMS. The presence of Sod1 within the IMS is dependent on Ccs1 (Field et al. 2003). Ccs1 contributes to the mitochondrial accumulation of Sod1 in addition to Cu(I) insertion.

Metallochaperones for Fe(II) and Mn(II) within the mitochondrial matrix may be expected as the two metals are redox active and can catalyze the Fenton reaction generating highly deleterious reactive oxygen species. Mitochondria contain its own genome that is susceptible to oxidative damage. Thus, metallochaperones for Fe(II) and Mn(II) may be important in controlling the reactivity of these ions together with ensuring their bioavailability and specific delivery. Iron donation to ferrochelatase and Isu1 for formation of heme and Fe-S cluster biogenesis, respectively, may involve the matrix protein frataxin (Lesuisse et al. 2003; Muhlenhoff et al. 2002). Consistent with these roles, Yfh1 interacts with both the Hem15 ferrochelatase and Isu1. Cells lacking the yeast frataxin (Yfh1) are heme deficient (Lesuisse et al. 2003) and compromised in Fe-S

cluster biogenesis (Muhlenhoff et al. 2002). However, frataxin is not essential for donating iron to ferrochelatase, as limited heme formation occurs in *yfh1*Δ cells. Likewise, depletion of yeast frataxin compromises but does not preclude Fe-S cluster biogenesis (Muhlenhoff et al. 2002). Frataxin adopts a well defined $\alpha\beta$-sandwich structural motif and is capable of binding Fe(II) ions in a N-terminal conserved acidic patch (He et al. 2004). Frataxin binds 2 Fe(II) ions as a monomeric protein with micromolar affinities (Cook et al. 2006). Thus, frataxin satisfies numerous criteria for being a non-essential iron metallochaperone in its ability to bind target molecules and iron in a transient manner.

No definitive metallochaperones have been identified in the mitochondrial matrix for manganese and zinc. In a screen for genes involved in Mn(II) uptake into mitochondria, a mitochondrial carrier protein designated Mtm1 was identified as a key component for metallation of Sod2 in the matrix (Luk et al. 2003a). Cells lacking Mtm1 are devoid of MnSod2 activity, but activity is restored by supplementation of cultures with high exogenous Mn(II) salts. Since mitochondrial Mn(II) levels are not depleted in *mtm1*Δ cells, the suggestion was made that Mtm1 had a role in Mn(II) trafficking to Sod2 within the matrix during the folding reaction (Luk et al. 2003a). Since metallation of Sod2 is coupled to protein folding, the metallation step may occur as the Sod2 polypeptide is emerging during import across the IM. Sod2 can be activated in the absence of Mtm1 in both the matrix and when redirected to the yeast cytoplasm in Mn-supplemented cells, so Mtm1 appears to function more in metal ion bioavailability (Luk et al. 2005). Mtm1 is a member of the MCF family of transporters and is predicted to be an amino acid carrier (Robinson and Kunji 2006). Thus, the import of the substrate of Mtm1 could conceivably help keeping Mn(II) bioavailable for Sod2 metallation.

Specificity of metallation reactions within the mitochondrion

An important question is how cells achieve specific metal ion insertion. Metal ion availability is a significant factor in the metallation of Sod2. The loss of

MnSod2 activity in *mtm1*Δ yeast cells is due to mis-metallation of Sod2 within the matrix (Yang et al. 2006). Cells lacking Mtm1 accumulate iron within the matrix and this iron preferentially metallates Sod2 leading to an inactive enzyme. Sod2 doesn't accumulate in the metal-free state, but prefers to exist in either the manganese or iron bound state. Mitochondrial iron pools are normally unavailable to Sod2, but when this pool is expanded in cells lacking Mtm1, iron preferentially binds to Sod2 rather than the catalytically active Mn(II) ion. The iron-inactivation of Sod2 in *mtm1*Δ cells is reversed by limiting iron availability to yeast or increasing cellular Mn(II) levels (Yang et al. 2006). Mitochondrial iron accumulation arising from loss of functions mutations in *SSQ1* or *GRX5* involved in Fe-S cluster biogenesis also results in an increase in bioavailable iron leading to mis-metallation of Sod2. However, mitochondrial iron accumulation from depletion of Yfh1 does not inactivate Sod2, and depletion of Yfh1 in cells lacking Mtm1 abrogates the iron-inactivation of Sod2. Thus, frataxin may mediate the iron-inactivation of Sod2.

Yeast lacking Yfh1 accumulate Zn-protoporphyrin IX (Lesuisse et al. 2003). The accumulation of Zn-protoporphyrin IX is also seen in Fe-deficient cells, suggesting that ferrochelatase can access Zn(II) pools within the mitochondrial matrix (Camadro and Labbe 1982; Labbe 1991; Zhang et al. 2005b). Thus, the iron substrate necessary for ferrochelatase is not supplied by an iron-specific metallochaperone, and metal ion availability is a component of the ferrochelatase reaction.

One test of the importance of metallochaperone-like molecules in metallation reactions is the expression of heterologous proteins in a cellular compartment. In the metallochaperone paradigm for metallation reactions, specific protein:protein interactions are a requisite for metal transfer. The prediction is that a heterologous metalloprotein would not be metallated in a nonnatural compartment. A series of heterologous proteins have been tested in yeast mitochondria. Yeast cells lacking MnSod2 are growth compromised in hyperoxia. The presence of matrix-targeted human CuZnSod1 in *sod2*Δ yeast allows the cells to propagate in hyperoxia implying that Sod1 is copper-activated

(Cobine et al. 2004). Expression of bacterial FeSod in the yeast matrix results in the presence of a functional Fe-enzyme. Bacterial or protistan FeSod targeted to the mitochondria of *sod2*Δ yeast cells protected the cells from the toxic effects of oxidative stress, whereas FeSod without a matrix targeting sequence failed to protect the cells (Balzan et al. 1995; Schott and Vasta 2003). Likewise, expression of plant alternative oxidase AOX in *S. cerevisiae* or *S. pombe* mitochondria leads to a functional diiron-containing enzyme that confers cyanide-resistant respiration. Metallation of all these heterologous proteins suggests that the mitochondrial matrix may not have an obligatory iron-metallochaperone and that pools of bioavailable iron and copper can be accessed by these matrix molecules. These studies suggest that pools of iron, copper and zinc exist within the mitochondrial matrix and can be utilized in nonphysiological and/or mis-metallation reactions. Regulation of the pool size of these metal ions must be an important process in eukaryotic cells to ensure proper metallation of each enzyme.

Metal ion pools within mitochondria

Mitochondria appear to have at least two pools of iron. One pool is bioavailable and is capable of competing with Mn(II) for the metallation of Sod2. This iron pool is expanded in yeast lacking Mtm1, Grx5 and Ssq1. Sod2 inactivation occurs in each of these strains (Yang et al. 2006). In contrast, cells lacking Yfh1 accumulate high levels of mitochondrial iron, yet this pool does not result in Sod2 inactivation. This second pool is less bioavailable, and preliminary evidence suggests that iron is predominantly insoluble Fe(III) (Lesuisse et al. 2003). Factors controlling the distribution of iron into these two pools are unknown, but one factor may be the presence of ligands that stabilize Fe(II) levels. Mutant cells with varying levels of a key matrix ligand may change the bioavailable pool of iron significantly. The identify of such Fe(II) ligands is under investigation. The prediction, that an amino acid is one substrate of the Mtm1 MCF exchanger (Robinson and Kunji 2006), suggests that *mtm1*Δ cells

accumulate an amino acid that stabilizes Fe(II). The fact that Fe(II), but not Mn(II), is more bioavailable in *mtm1*Δ cells suggests that whatever the putative ligand that stabilizes Fe(II) has less effect on Mn(II) availability (unpublished observation).

Other yeast MCF proteins modulate mitochondrial metal ion pools. Ggc1 (also known as Yhm1) is important for iron homeostasis. Yeast lacking Ggc1 accumulate iron in mitochondria and show impaired heme synthesis (Lesuisse et al. 2004). These phenotypes may imply a direct role in mitochondrial iron transport, yet Ggc1 was shown to be a GTP/GDP carrier important in mitochondrial GTP uptake (Vozza et al. 2004). The relationship of GTP/GDP exchange and iron pools within the mitochondrion remains unclear, but GTP/GDP exchange activity in Ggc1-reconstituted vesicles is inhibited by an iron chelator (Vozza et al. 2004). Sam5 is a mitochondrial carrier for S-adenosylmethionine (SAM) (Marobbio et al. 2003). Cells lacking Sam5 are biotin auxotrophs as biotin synthesis requires the SAM-dependent enzyme Bio2 within the mitochondrial matrix. In addition, we have preliminary evidence that *sam5*Δ cells are deficient in mitochondrial copper.

Zn(II) pools within the matrix are uncharacterized, but this pool must be significant as numerous Zn-binding metalloenzymes exist within this organelle. A mitochondrial Zn(II) pool was reported in neurons that can be pharmacologically mobilized during neuronal injury observed in cerebral ischemia (Sensi et al. 2003).

Mitochondria contain a pool of copper that is distinct from that associated with CcO and Sod1. A small fraction of mitochondrial copper is associated with these cuproproteins. The bulk of the remainder is localized within the matrix as a soluble, low molecular weight ligand complex. The CuL complex is conserved in the mitochondria of yeast and mouse cells. In yeast and mouse liver 85 and ~70% of the total mitochondrial copper is associated with this matrix CuL complex. Although the identity of the ligand remains unresolved, the matrix CuL pool is accessible to a heterologous cuproenzyme (Cobine et al. 2004). Localization of human Sod1 within the mitochondrial matrix results in the

suppression of the growth defect of *sod2*Δ cells. Targeting two different Cu-binding proteins, human Sod1 and Crs5, to the mitochondrial matrix but not the cytoplasm, results in cell growth impairment on non-fermentable carbon sources and decreased levels of CcO activity. These effects are reversed by supplementation of cells with $CuSO_4$ to the culture medium. In addition the presence of the matrix targeted Crs5 results in diminished Sod1 protein levels within the IMS and impaired activity of an IM-tethered hSod1. Copper binding by the matrix-targeted proteins attenuates the level of the CuL complex within the matrix without affecting the total mitochondrial copper levels suggesting that biochemical attenuation of the matrix CuL complex via heterologous competitor molecules limits the available copper for redistribution to the IMS for metallation of CcO and Sod1. The CuL pool appears to be used in the metallation of CcO and Sod1 within the IMS.

Role of mitochondria in controlling metal pools in cell

The mitochondrion has a central role in modulating iron homeostasis in yeast. Iron homeostasis in *S. cerevisiae* is largely regulated at the level of transcription via the iron-responsive transcriptional activators Aft1 and Aft2. The set of genes regulated by Aft1 and Aft2 includes a series of genes whose products function in iron acquisition and intracellular iron distribution. Iron-regulation of Aft1 function is regulated in part through the localization of Aft1. Aft1 is active in Fe-deficient cells as a nuclear factor, and inactive in Fe-replete cells where it is diffusely localized within the cytoplasm (Yamaguchi-Iwai et al. 2002). The mechanism by which Aft1 (and Aft2) sense cellular iron levels involves the mitochondrion (Chen et al. 2004; Chen et al. 2002). Cells defective for Fe-S cluster biogenesis within the mitochondrial matrix exhibit constitutive expression of the iron regulon genes (Chen et al. 2002). The constitutive activity of Aft1 is due to an impairment of a signal arising from the mitochondrial Fe-S biosynthetic machinery and not due to Fe-deficiency within the cytoplasm (Chen et al. 2004). Iron sensing by Aft1 requires proper mitochondrial Fe-S cluster biosynthesis, as

well as the export of an ill-defined intermediate through the Atm1 IM transporter (Rutherford et al. 2005). However, Aft1 iron sensing does not require the cytoplasmic machinery to mature Fe-S clusters (Rutherford et al. 2005), demonstrating that iron sensing by Aft1 is not linked to the maturation of cytosolic 4Fe-4S clusters.

The cytoplasmic monothiol glutaredoxins 3 and 4 are important in iron sensing by Aft1 (Ojeda et al. 2006). Cells lacking Grx3 or Grx4 have a constitutively active Aft1 residing in the nucleus. Glutaredoxins are glutathione-dependent, thiol-disulfide oxidoreductases that function in maintaining the cellular redox homeostasis. Monothiol glutaredoxins are believed to reduce mixed disulfides formed between a protein and glutathione in a process known as deglutathionylation. The only other monothiol glutaredoxin in yeast is Grx5 that is localized within the mitochondrial matrix where it participates in the maturation of Fe-S clusters (Rodriguez-Manzaneque et al. 2002). Grx3 and Grx4 are predominantly localized to the nucleus (Belli et al. 2002), and they both interact with Aft1 in an iron-independent manner (Ojeda et al. 2006). We postulate that the interaction of Aft1 and Grx3/Grx4 within the nucleus preconditions Aft1 to sense the inhibitory signal emanating from the mitochondrion. The inhibitory signal is postulated to cause a conformational change in Aft1 that destabilizes DNA binding and induces nuclear export of Aft1. A major question remaining is the identity of the iron-inhibitory signal exported from the mitochondrion by Atm1.

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