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Labile Low-Molecular-Mass Metal Complexes in Mitochondria: Trials and Tribulations of a Burgeoning Field

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Abstract

Iron, copper, zinc, manganese, cobalt, and molybdenum play important roles in mitochondrial biochemistry, serving to help catalyze reactions in numerous metalloenzymes. These metals are also found in labile “pools” within mitochondria. Although the composition and cellular function of these pools are largely unknown, they are thought to be comprised of nonproteinaceous low-molecular-mass (LMM) metal complexes. Many problems must be solved before these pools can be fully defined, especially problems stemming from the lability of such complexes. This lability arises from inherently weak coordinate bonds between ligands and metals. This is an advantage for catalysis and trafficking, but it makes characterization difficult. The most popular strategy for investigating such pools is to detect them using chelator probes with fluorescent properties that change upon metal coordination. Characterization is limited because of the inevitable destruction of the complexes during their detection. Moreover, probes likely react with more than one type of metal complex, confusing analyses. An alternative approach is to use liquid chromatography (LC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). With help from a previous lab member, the authors recently developed an LC–ICP-MS approach to analyze LMM extracts from yeast and mammalian mitochondria. They detected several metal complexes, including Fe₅₈₀, Fe₁₁₀₀, Fe₁₅₀₀, Cu₅₀₀₀, Zn₁₂₀₀, Zn₁₅₀₀, Mn₁₁₀₀, Mn₂₀₀₀, Co₁₂₀₀, Co₁₅₀₀, and Mo₇₈₀ (numbers refer to approximate masses in daltons). Many of these may be used to metalate apometalloproteins as they fold inside the organelle. The LC-based approach also has challenges, e.g., in distinguishing artifactual metal complexes from endogenous ones, due to the fact that cells must be disrupted to form extracts before they are passed through chromatography columns prior to analysis. Ultimately, both approaches will be needed to characterize these intriguing complexes and to elucidate their roles in mitochondrial biochemistry.

Graphical abstract

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Redox-active transition metals, including iron, copper, manganese, cobalt, and molybdenum (as well as redox-inactive zinc), play critical roles in mitochondrial biochemistry. They are typically bound at the active sites of enzymes where they help catalyze reactions. Their excellent catalytic properties derive from (a) the availability of d orbitals to participate in redox chemistry and bonding, (b) the abundance of coordination sites that can accommodate and orient multiple substrates, and (c) the weakness of coordinate bonds that promotes facile binding and release of substrates, intermediates, and products. Simple monodentate ligands tend to exchange rapidly when coordinated to first-row d-block metal ions. For example, aqueous Mn^{II} , Fe^{II} , Co^{II} , Cu^{II} , and Zn^{II} complexes have water-exchange rates ranging from 10^6 to 10^9 s^{-1} .¹ Unfortunately, this lability makes isolating and characterizing such complexes difficult. Ligand-exchange kinetics are slowed dramatically when metals coordinate to large polydentate ligands (e.g. protein binding sites). As a result, most metals bound in proteins are relatively inert except for the open coordination sites to which substrates bind. Nonproteinaceous LMM metal complexes tend to be more labile, though there are exceptions to this tendency. For instance, metals that are bound to metallochaperone carrier proteins must be sufficiently labile to facilitate delivery to downstream intracellular targets. Also, some metalloenzymes contain labile metal centers.² Other exceptions are siderophores, LMM organic chelating agents secreted by certain microorganisms to sequester trace amounts of Fe^{III} from the environment.³ These LMM iron complexes are generally inert, except at low pH. In this review, we focus on labile LMM metal complexes, defined here as <10 kDa, that are, for the most part, nonproteinaceous.

The discovery of metallochaperone proteins in the 1990s suggested that upon entering the cell, transition metals are transported to their respective target proteins by being passed from one metallochaperone to another, like a baton handed from one runner to the next in a relay race. In this way, aqueous metal complexes (sometimes erroneously called “free” metal ions) or other nonproteinaceous metal complexes were not thought to exist in the cell.^{4–6} This

view has evolved somewhat in recent years because of the detection of labile metal pools within cells. Although their exact cellular functions are typically unknown, these pools likely participate in cellular trafficking, regulation, signaling, and/or storage of metal ions.^{7,8} Given their lability (and low concentrations in cells), characterizing the structures and functions of the metal complexes that compose these pools has been, and continues to be, a considerable challenge.

In this review, we focus on labile LMM metal complexes located specifically in mitochondria. These organelles are major “traffic hubs” for an array of transition metals. Mitochondria contain respiratory complexes and respiration-related proteins that are packed with iron–sulfur clusters (ISCs) and heme centers. In fact, both ISC assembly and the Fe insertion step of heme biosynthesis occur within this organelle.⁹ Mitochondria house Cu-containing proteins such as cytochrome *c* oxidase, Cu/Zn superoxide dismutase (Sod1), and the many chaperones that shuttle Cu to these two enzymes during their assembly.^{10,11} Mitochondria house an important manganese-containing enzyme, namely superoxide dismutase (MnSod2).¹² Mammalian mitochondria also contain arginase II, a dimanganese enzyme.^{13,14} Approximately 15% of the Co in mammalian cells is found in mitochondria,¹⁵ most of which is bound at the active site of methyl-malonyl-CoA mutase (MUT). A small portion of mitochondrial Co is coordinated to trafficking proteins that install adenosylcobalamin into apo-MUT. Mammalian mitochondria contain three molybdenum enzymes, including sulfite oxidase and mitochondrial amidoxime reducing component isoforms 1 and 2 (mARC1 and -2, respectively).¹⁶

Mitochondria house numerous Zn-containing proteins, the best known of which include Cu/Zn superoxide dismutase (Cu/Zn Sod1) and cytochrome *c* oxidase.^{17,11,18} In yeast, alcohol dehydrogenase III is a Zn enzyme that is involved in NAD(P)H redox balance and the ethanol–acetaldehyde shuttle under anaerobic conditions.^{19,20} D-Lactate dehydrogenase is a Zn-containing enzyme that catalyzes the oxidation of lactate to pyruvate.²¹ The Zn enzyme glyoxalase II promotes the hydrolysis of *S*-D-lactoylglutathione to glutathione and D-lactate in the matrix.²² α -Isopropylmalate synthase I and II are Zn enzymes that catalyze the first step of leucine biosynthesis.²³ Both of these enzymes are regulated by the Zn-dependent reversible inactivation by coenzyme A, which links leucine biosynthesis to mitochondrial energy metabolism.²⁴ Zn-bound MST1 is a mitochondrial threonyl-tRNA synthetase that aminoacylates two tRNAs.²⁵

Virtually all mitochondrial proteins are encoded in the nucleus, synthesized on ribosomes in the cytosol, and imported into mitochondria via protein translocation machines.^{26,27} Many nascent unfolded proteins are threaded through the TOM (translocase of the outer mitochondrial membrane) complex and then sorted according to their final intramitochondrial destinations.²⁸ The imported proteins that are targeted for the matrix typically contain a presequence that is cleaved by mitochondrial processing peptidase (MPP), a reaction that triggers protein folding. Numerous Zn-containing metalloproteases are involved in these processes. Mas1/2 is the MPP metalloendopeptidase that cleaves presequences from the majority of imported mitochondrial proteins.²⁹ Oct1 is a matrix intermediate metallopeptidase that cleaves destabilizing N-terminal residues of some proteins after cleavage by MPP.³⁰ Afg3 is a subunit of the ATP-dependent m-AAA

metalloprotease found in the IM.²⁹ Yme1 is the Zn-containing catalytic subunit of the i-AAA metalloprotease complex; this IM enzyme helps degrade unfolded or misfolded mitochondrial proteins.³¹ Cym1 and Prd1 are lysine-specific metalloproteases that degrade proteins and presequences.³⁰ Other Zn-containing proteins are involved in protein import or folding. ZIM17 (zinc finger motif protein of 17 kDa) helps import and fold proteins entering the matrix.³² MDJ1 stimulates the ATPase activity of the Hsp70 protein Ssc1p and helps fold or refold proteins in the matrix. This Zn-containing protein helps form mitochondrial nucleoids and maintain mtDNA stability.³³ Zn-containing TIM9 and TIM10 form a hexameric complex in the IMS that delivers hydrophobic proteins to the TIM22 complex for insertion into the IM.³⁴ Binding of Zn to Cys residues stabilizes these proteins and prevents oxidation to disulfides. Newly imported IMS proteins TIM10, TIM12, and TIM13 bind Zn and interact with Mia40 for folding and oxidation.³⁵ Mia40 is an IMS protein that coordinates Zn.³⁶ The Zn bound to these small TIM proteins may be transferred onto Mia40 during oxidation (a process that traps these proteins in the IMS). Zn bound to reduced Mia40 may inhibit its reoxidation by Erv1. IMS protein Hot13 may remove Zn from Mia40 to aid in its reoxidation.³⁷ However, the small TIM proteins may not be importable with Zn bound, suggesting an alternative role for Hot13.³⁸

The folding of nascent apo-metalloproteins in the matrix is typically accompanied by the incorporation of metal ions or centers. The question is how these metals reach the matrix. After all, the IM is notoriously impermeable such that even protons are unable to diffuse across it under normal conditions. In all likelihood, metal ions enter the matrix through specific transport proteins on the IM.^{11,39} Similar issues arise for metalloproteins targeted to other mitochondrial locations; specific metal complexes must be used, and there must be distinct pathways that guide them to the appropriate location within the organelle so that they can metalate their targets. Thus, metalation reactions in the IMS are likely different from those in the matrix, which may be especially important for Zn and Cu trafficking.

Metals en route to mitochondria must first pass through the OM. For LMM complexes, this may involve simple diffusion through the hydrophilic pores of VDACs (voltage-dependent anion channels). These porins constitute ~30% of the OM surface area,⁴⁰ creating pores with diameters of 2–3 nm.⁴¹ Species with masses of less than ~800 Da, including water, ions, ATP/ADP, and perhaps LMM metal complexes, can freely diffuse through VDACs, whereas import of larger compounds is regulated. In yeast, VDAC pores are regulated by the metabolic transition from fermentation to respiration.⁴²

Two general strategies, in combination with genetic and spectroscopic methods, have been used to investigate labile metal pools in mitochondria. The most popular entails custom-designed chelators that are localized to mitochondria and bind specifically (or at least preferentially) to a particular metal. Fluorescence associated with these probes or sensors either increases, decreases, or shifts upon metal coordination. Chelator-based sensors must be hydrophilic enough to be soluble in aqueous regions of the cell, yet lipophilic enough to traverse membranes. Satisfying these criteria is a formidable endeavor that requires considerable synthetic abilities.

The alternative strategy to studying labile LMM metal pools is to isolate individual complexes using liquid chromatography (LC). Unfortunately, these metal species are difficult to isolate because of their lability and low concentrations. There is also a lack of commercially available technologies (e.g., chromatography columns) designed to help isolate LMM metal complexes from biological extracts. Our strategy has been to assemble a Bioinert LC instrument inside a refrigerated anaerobic glovebox and to split the eluate from size-exclusion columns such that a portion is collected in fractions and the remainder flows to an online inductively coupled plasma mass spectrometer (ICP-MS) (Figure 1). Employing this experimental system allows LMM metal complexes to be detected, isolated, and characterized. This approach is limited to metal complexes that do not decompose during chromatographic workup. Evidence of decomposition would include chromatography peaks corresponding to the masses of aqueous ("free") metal ions (<200 Da). Although anaerobic conditions tend to retain metals in their reduced oxidation states, some oxidation might still occur.

In our most recent LC-ICP-MS study,⁴³ we isolated mitochondria from fermenting yeast, human Jurkat cells, mouse brain, and mouse liver. Purified mitochondria were treated with detergent, and the soluble supernatant fraction was passed through a 10 kDa cutoff membrane in an Amicon (Merck Millipore Corp.) stirred cell. The flow-through solution (FTS) was injected onto a size-exclusion column that could resolve metal complexes 10 kDa in size. The approximate mass of any observed species was estimated (to $\pm 30\%$) by calibrating the column with a series of standards of known molecular masses. Online ICP-MS monitored Fe, Cu, Zn, Mn, Co, Mo, S, and P; phosphorus traces were used as internal standards. Although sufficiently stable to remain intact down an LC column, the observed metal-containing species were sufficiently labile to be affected by treatment with 1,10-phenanthroline (Phen).⁴³

Both approaches have contributed valuable insights to our current understanding of labile metal pools in biological systems, yet each has shortcomings. A significant limitation of the chelator-based method is that the ligands associated with an endogenous metal complex are inevitably displaced when the probe coordinates the metal. Thus, chemical characterization of such complexes has not been possible and does not seem possible moving forward. Moreover, a given chelator will almost certainly interact with numerous labile metal species, perhaps including sites that are typically considered nonlabile, e.g., metalloprotein active sites. Different chelators (or even the same chelator reacting under different conditions) undoubtedly detect different labile metal pools. Some of these problems have been demonstrated recently with Zn sensors,⁴⁴ and other fundamental questions remain unanswered. For example, do probes targeted to mitochondria interact with labile metal centers in the cytosol en route to their target? Might the resulting metal-bound probes enter mitochondria and thus be counted (erroneously) as evidence of a mitochondrial pool? Is the concentration of the chelator that ultimately localizes to the mitochondria sufficient such that the endogenous concentration of labile metal (rather than of the chelator itself) dictates the extent of reaction and thus the observed pool size? Many of these questions have been raised,^{43,44,7} but they must be addressed experimentally before we can have confidence in the results obtained by this approach.

A major drawback of the chromatography-based strategy is that the structural integrity of cells and organelles must be destroyed during the isolation of LMM metal complexes. As a result, cellular contents are mixed together, which can promote side reactions involving these labile metals, giving rise to artifacts. Thus, every LC peak in the chromatograms shown below (from our lab, no less!) might be an artifact of the isolation procedure (however, we doubt that this is the case). Another problem is that isolated mitochondria may not be pure, and contaminating species from other cellular compartments can confuse analyses. Typically, the purity of these organelles is assessed by microscopy and Western blots. Ascertaining the cellular function of a metal complex that has been isolated from the cell is difficult. However, additional information can be obtained by fractionating mitochondria into subcomponents.⁴⁵

Overall, the chelator-based strategy may be better suited to address functional aspects of labile LMM metal complexes, whereas the chromatography-based approach may be better suited for cataloging and chemically characterizing individual metal complexes. Now cognizant of these strengths and weaknesses, we are ready to discuss recent discoveries and current controversies surrounding labile metal complexes in mitochondrial biochemistry.

IRON

The most prominent mitochondrial transporters of transition metals are mitoferrins 1 and 2 (Mrs3/4 in yeast). These homologous proteins are high-affinity iron transporters situated in the IM.^{46–49} They are members of the mitochondrial carrier family (MCF), which includes 53 proteins in humans and 35 proteins in yeast.⁵⁰ The physiological differences between mitoferrin 1 and 2 (or Mrs3/4) pairs have yet to be fully established. Mitoferrin 1 is an essential iron importer for developing erythrocytes, whereas mitoferrin 2 may be required for heme and ISC assembly in nonerythroid cells.⁵¹ The iron transported through these high-affinity importers is ultimately utilized in the biosynthesis of ISC and heme cofactors.^{52,53} Indeed, the majority of Fe that accumulates in mitochondria of ISC mutants (e.g., frataxin-deficient cells) passes through these carrier proteins.

Rim2, another MCF protein, translocates Fe ions and pyrimidine nucleotides.^{54,55} In the absence of Mrs3/4, Rim2 has been shown to mediate the import of Fe into the matrix. Interestingly, the extent of ISC biogenesis is diminished in yeast cells that lack Mrs3/4 and Rim2 but not in cells lacking only Rim2. This implies that Rim2 is a backup low-affinity Fe importer. The non-lethality of the Mrs3/4 and Rim2 triple deletion strain implies an additional route of Fe import into mitochondria.

The Fe complexes that are recognized by these mitochondrial import proteins have not been identified. In general, MCF members transport a variety of chemically diverse substrates across the IM, such as nucleotides, amino acids, keto/carboxylic acids, phosphate ions, and cofactors. Although the structures of many MCF members, including the mitoferrins, have not been determined, their sequences are similar enough that an approximate structure can be estimated. Mitochondrial carrier proteins have basket-shaped structures. The bottoms of these structures face the matrix side and are structurally stabilized by salt bridge networks. They have a common substrate binding site located at the midpoint of the IM.⁵⁶ Mitoferrins

possess three conserved His ligands that are spatially arranged like steps of a spiral staircase to shuttle Fe^{II} ions across the membrane,⁵⁷ along with three conserved Asp/Glu residues that may participate in metal transport. Mitoferrins do not belong to any established class of MCF proteins, so their substrates are unlikely to be similar to those listed above. Nevertheless, their substrates must be small enough to fit through the narrow channels in these proteins. This excludes large metallochaperones from consideration as substrates and suggests that LMM metal complexes may serve such roles.

An early study by Tangerås et al.⁵⁸ identified a labile Fe pool in mitochondria isolated from rat livers. In their study, extracts were treated with bathophenanthroline sulfonate, a chelating agent that turns red when coordinated to Fe^{II}, thereby permitting quantification of the labile Fe^{II} concentration in their samples. Their findings suggest that ~25% of mitochondrial Fe is labile and primarily located in the matrix. In a later study, Petrat et al.^{59,60} used membrane-permeable chelators to detect and quantify a labile Fe pool within mitochondria of intact whole cells. They incubated cells with fluorescent probes that penetrated into mitochondria and quenched in response to binding Fe. Subsequent treatment with a more powerful chelator removed all of the Fe bound to the sensor, concomitantly restoring the fluorescent signal. The difference in emission was then quantified, affording labile Fe concentrations of 5–17 μM in rat mitochondria.⁶¹ Mitochondria contain ~700 μM Fe,⁶² so the labile Fe fraction, as quantified, corresponds to 1–2% of mitochondrial iron, far less than what was determined by Tangerås and co-workers. Petrat et al. postulated that the higher concentration reported in the earlier study was artifactual.^{59,60}

More recently, we examined mitochondria isolated from fermenting and respiring yeast cells using Mössbauer spectroscopy,⁶² which can distinguish various groups of Fe-containing species, including hemes, ISCs, and non-heme Fe. We discovered that ~20% of the iron in fermenting mitochondria (~150 μM) is present as non-heme high-spin (NHHS) Fe^{II}.⁶³ Membrane-impermeable chelators were unable to access this pool of iron unless mitochondria were disrupted by sonication or treated with detergent. By contrast, membrane-permeable Phen selectively chelated the NHHS Fe^{II} ions without perturbing the other Fe-containing species in the organelle. In independent studies, Lutz et al.⁶⁴ and Pandey et al.⁶⁵ developed assays for monitoring ISC biogenesis in intact mitochondria. These investigators found that treating isolated mitochondria with Phen inhibits ISC activity. They concluded that mitochondria contain a pool of labile Fe that is used as feedstock for ISC assembly. When considered with our Mössbauer results showing that NHHS Fe^{II} is selectively chelated by phen, it becomes evident that this pool of NHHS Fe^{II} is used as a feedstock for ISC assembly and that it is probably transported through mitoferrin carriers.

The same pool might also be feedstock for heme biosynthesis, but further studies are required to establish this. Lange et al. concluded that Fe in the matrix is not used for insertion into porphyrin and that Fe is supplied to ferrochelatase directly from the IM.⁶⁶ However, the subsequent structure of ferrochelatase revealed that the Fe^{II} binding site is exposed to the matrix, implying that heme Fe is contributed by a pool in this subcompartment.⁶⁷ Dancis and co-workers performed experiments demonstrating that most Fe^{II} ions used in heme biosynthesis enter the matrix through the IM mitoferrins, Mrs3/4.⁵²

Our current research efforts are focused on characterizing the endogenous iron species that constitute the mitochondrial labile iron pool. In our most recent LC-ICP-MS study, we found that FTSs of mitochondrial extracts from fermenting yeast contain either of two LMM Fe complexes (Figure 2), depending on whether cells are harvested in the exponential or postexponential growth phase.⁴³ In the former case, a LMM species with an estimated mass of 580 Da (called Fe₅₈₀) is present, whereas in the latter, a complex corresponding to a mass of 1100 Da (Fe₁₁₀₀) is observed. Curiously, Fe₁₁₀₀ converts into Fe₅₈₀ when the FTS is allowed to incubate inside an anaerobic glovebox for a few days, suggesting that these two compounds are somehow related. Their combined concentration in mitochondria is calculated to be ~100 μ M, consistent with the estimate of Tanagerås et al.⁵⁸ and with the quantified intensity of the NHHS Fe^{II} species observed by Mössbauer.⁶³ Fe₅₈₀ may be the NHHS Fe^{II} species that is transported through Mrs3/4. In studies of fermenting mitochondria harvested during exponential growth on medium that contained a 10-fold excess of Fe^{III} citrate (100 μ M rather than 10 μ M), the intensity of the Fe₅₈₀ peak increased 1.7-fold (Figure 2, top left panel, 10x), while the peak intensities of other metals (see below) were unaffected. FTSs from mammalian mitochondria also contain Fe₅₈₀ and Fe₁₁₀₀ as well as an iron species with a mass of ~1500 Da (Fe₁₅₀₀). This illustrates a general theme that LMM metal complexes in yeast mitochondria are also found in mammalian mitochondria, but mammalian mitochondria might contain additional LMM metal complexes. Finally, treatment of FTSs with Phen causes widespread changes in the metal chromatograms, demonstrating that the observed LMM metal complexes are, in fact, labile.

The coordination chemistry of iron and glutathione (GSH) may be relevant to this issue. GSH represents ~90% of cellular LMM sulfur-containing species,⁶⁸ with 10–15% of that located in mitochondria.⁶⁹ The concentration of GSH in mitochondria is very high (10–14 mM).^{70,71} Hider and Kong argue that an Fe^{II}(GSH) adduct is the major LMM Fe complex in the cytosol based on a high stability constant for the complex and the abundance of GSH within the cytosol.⁷² A similar argument could be made for LMM Fe^{II}(GSH) adducts in the mitochondrial matrix. Citrate, another metabolite present in the matrix at high concentrations, should also be considered as a potential ligand for LMM metal complexes.^{71,73}

Another aspect of iron:GSH metabolism occurring in yeast mitochondria involves the IM protein Atm1 (ABCB7 in humans). Atm1 exports a poorly defined LMM sulfur-containing species called X-S, which is used as feedstock for ISC assembly in the cytosol and as a signaling molecule that regulates the import of iron into the cell and mitochondria. The Atm1 structure has either GSH, GSSG (glutathione disulfide), or GSSH (glutathione peroxide) bound, suggesting that it catalyzes the export of these or similar molecules (e.g., the trisulfide GSSSG) into the cytosol.^{74,75} GSH also coordinates Fe₂S₂ clusters, and the [Fe₂S₂(GS)₄]²⁻ complex has been proposed to be present in mitochondria and exported by Atm1.⁷⁶ Further studies are required to determine whether any of the labile LMM Fe species in mitochondria involve GSH coordination, but the masses of Fe₅₈₀ and Fe₁₁₀₀ are within error of those of Fe^{II}(GSH)(OH)₂ and [Fe₂S₂(GS)₄]²⁻, respectively. Evidence that this is more than mere coincidence is required before any such assignment should be considered further.

COPPER

Initial studies by Winge, Cobine, and co-workers concluded that 70–85% of mitochondrial Cu is associated with a LMM complex found in the matrix called CuL.^{77,78} An elaborate isolation procedure is employed to purify CuL. Cellular extracts are treated with 100% methanol. The resulting extracts are dried and resuspended in aqueous solutions before being loaded onto an anion-exchange column. Fractions containing the CuL apo ligand are dried and resuspended in water prior to injection onto a C18 reverse-phase column. Ligand-containing fractions elute in the middle of a methanol gradient and are subsequently screened for fluorescence.⁷⁹ Cu^I is added to fractions containing the purified apo ligand, resulting in the formation of CuL as indicated by fluorescence quenching. The material obtained from this rather involved procedure, defined as CuL, is soluble, anionic, and stable to boiling and migrates on size-exclusion columns like a globular protein with a mass of 13,000 Da.⁷⁷ However, CuL exhibits no absorption at 280 nm and is unaffected by proteinase K treatment, suggesting that it is nonproteinaceous. In terms of biological significance, Winge and Cobine view the CuL pool as a reservoir or “dynamic rheostat” that is used to buffer intracellular copper levels. They hypothesize that the Cu in CuL is ligated by small metabolites such as organic acids or nucleotides. They suggest that the CuL ligand prevents mitochondrial Cu from damaging ISC that are generated in the matrix.

These researchers hypothesize that CuL is originally produced in the cytosol and imported into the matrix by Pic2⁷⁹ and Mrs3,⁸⁰ both of which are MCF members. They conclude that Pic2 mediates the transport of Cu into the matrix, which is supported by the observation that *pic2* cells exhibit a growth phenotype on synthetic Cu-deficient non-fermentable medium (whereas growth on Cu-sufficient media shows no phenotype). Moreover, increasing the amount of silver ions in the growth medium, which directly compete with Cu ions, exacerbates the growth defect. The rate of Cu uptake in *pic2* mitochondria is nearly half that for wild-type (WT) mitochondria. Consistent with this, the concentration of Cu within mitochondria from *pic2* cells is only ~70% relative to that in WT mitochondria. Together, these results infer the existence of an alternative mitochondrial Cu transport protein, presumably Mrs3.⁸⁰

Other aspects of mitochondrial copper metabolism occur in the IMS.⁸¹ During assembly of cytochrome *c* oxidase, Cu is transported into the IMS where it is incorporated into the Cu_B and Cu_A sites of the enzyme using a variety of required ancillary proteins. Metalation of Cu/Zn Sod1 also occurs within the IMS and is mediated by the Cu chaperone Ccs1.¹² The form of Cu that enters the IMS and the compartment from which it enters remain unresolved. Winge and Cobine conclude that CuL is shuttled into the IMS from the matrix by an unidentified IM carrier.^{78,80} However, with a mass of 13,000 Da, CuL is too large to diffuse through a carrier protein channel.⁸² Another possibility is that Cu from the cytosol is directed into the IMS by Cox17, a soluble 8 kDa protein containing a Cu^I center coordinated by three conserved cysteines.^{83–87} Indeed, this protein is found in both the IMS and cytosol.⁸⁵ However, Cox17 is translocated into the IMS by the Mia40 oxidative folding pathway, meaning that it arrives in the IMS as an unfolded apoprotein.^{88,89} Moreover, Maxfield et al. demonstrated that tethering Cox17 to the IM affords normal cytochrome *c* oxidase activity,⁹⁰ indicating that it does not import Cu from the cytosol. The Cu trafficking

pathway downstream of Cox17 is better characterized. Cox17 supplies Cu to Cox11 (34 kDa) and Sco1 (33 kDa) through specific protein–protein interactions. Other small soluble Cu binding IMS proteins involved in the metalation of cytochrome *c* oxidase include Cox19 (11 kDa) and Coa6 (12 kDa).^{91,92}

Labile Cu pools in mitochondria have been detected by chelator-based sensors. Yang et al.⁹³ used a membrane-permeable copper-selective fluorescent probe along with X-ray fluorescence microscopy to detect and characterize a labile pool of low-coordinate Cu^I with sulfur-based ligands in mitochondria of fibroblasts that had been pretreated with a high concentration of CuCl₂. Cu-based signals were nearly undetectable in samples that were not treated in this manner, which raises concern that newly imported copper arising from the Cu treatment was detected, rather than endogenous Cu in the organelle. Nevertheless, the authors concluded that their results “strongly support” the presence of an endogenous labile Cu pool within the mitochondrial matrix. In this case, substantial amounts of the Cu matrix pool should have also been found in mitochondria of cells grown on medium that was not spiked with Cu, a condition for which Yang et al. detected little Cu intensity.⁹³

Better evidence of endogenous labile Cu in mitochondria is provided by Dodani et al.,⁹⁴ who similarly employed a mitochondrially targeted Cu sensor to detect labile copper pools within the organelle. Fluorescence increased 34% in live, intact cells that had been incubated in high concentrations of CuCl₂. Conversely, emission decreased 36% after a strong Cu chelator had been added, which presumably entered the mitochondria and coordinated endogenous Cu in the organelle. Giuffrida et al. developed a water-soluble highly selective fluorescent Cu^I probe that is specific for mitochondria.⁹⁵ No significant effects on cell viability were observed after neuroblastoma cells had been treated with large doses of this chelator. This confirms that the sensor does not damage the cell, but it also raises doubts that it chelates a form of Cu that is physiologically relevant.

With the help of our LC–ICP–MS system, we searched for CuL by examining FTSs of mitochondrial extracts that had been filtered through a 10 kDa membrane.⁴³ Only one LMM Cu species, with a molecular mass of ~5000 Da (Cu₅₀₀₀), was reproducibly observed in yeast mitochondria (Figure 2). We did not detect a major Cu-containing peak at 13 kDa, nor did we consistently observe any intense Cu features with masses of <5 kDa. We did observe some minor intensity peaks in the very low-mass region, but their elution volumes were not reproducible from batch to batch. We suspect that these minor species are artifacts. Given that our samples were passed through a 10 kDa cutoff membrane, an argument could be made that we should not have observed CuL in our experiment. However, 10 kDa membranes do not completely restrict passage of entities with masses slightly greater than 10 kDa; indeed, we have detected peaks with masses as high as 20 kDa in the FTS. Besides having a mass that is significantly different from that of CuL, Cu₅₀₀₀ accounts for only ~20% of Cu in yeast mitochondria, whereas CuL reportedly accounts for 70–85% of mitochondrial Cu. Also, we have not observed Cu₅₀₀₀ in mammalian mitochondrial extracts, whereas CuL has been reported to be present in both yeast and mammalian mitochondria.³⁹

In summary, we are currently unable to rationalize all of the published results involving labile LMM Cu species in mitochondria using a single, self-consistent model. There is

consensus about the following: At least 20% of mitochondrial Cu is labile and LMM; labile Cu in mitochondria can be detected by chelator-based fluorescent probes; a portion of labile and LMM Cu is located within the IMS. There is disagreement about whether labile/LMM mitochondrial Cu represents ~20 or ~80% of total mitochondrial Cu, is mostly located in the matrix or IMS, enters the IMS from the cytosol via the OM or from the matrix via the IM, and has a mass of 13,000 Da, 5000 Da, or not more than ~1000 Da.

If transported Cu enters the matrix and then passes into the IMS, it should have a mass of not more than ~1000 Da because larger Cu-bound species are unable to fit through the channels of IM transporters. Similarly, if transported Cu enters the IMS directly from the cytosol, it should be small enough to diffuse through the VDAC pores of the OM. Conceivably, Cu₅₀₀₀ might pass through these pores when they are open. Further studies are needed to resolve these issues.

ZINC

Wing, Eide, and co-workers have also detected and isolated a cationic LMM Zn pool in mitochondrial extracts.⁹⁶ The pool is stable to boiling and proteinase K digestion, suggesting that it is not proteinaceous. Under Zn-sufficient growth conditions, approximately half of mitochondrial Zn is soluble, and a significant portion of this comprises the Zn pool. When cells are grown on high-Zn medium, the size of the pool increases significantly. Reducing the size of the pool (by genetically inserting a Zn-requiring alcohol dehydrogenase apoprotein into the matrix, which then incorporates Zn from the pool during metalation) causes cells to have difficulty respiring. This suggests that the Zn pool is required for respiration.

Labile Zn pools in mitochondria have also been identified using fluorescent probes. Tomat et al.⁹⁷ synthesized a Zn-sensitive chelator that was targeted to the mitochondria of HeLa cells. In their study, they observed a dramatic increase in fluorescence intensity after cells were treated with 10–50 μM ZnCl₂. Sensi et al. detected a pool of labile Zn ions in mitochondria from mammalian neuronal cells.⁹⁸ This pool was in dynamic exchange with a labile cytosolic Zn pool, and its size increased in cells grown on medium supplemented with Zn.^{98,99} High levels of cytosolic Zn induce loss of mitochondrial membrane potential (Ψ_m), perhaps by opening IM channels or pores. Malaiyandi et al. used sensors to monitor the uptake of exogenously added Zn in the matrix of mitochondria isolated from rat brains.¹⁰⁰ Zn²⁺ was imported via a Ca²⁺ uniporter in a process that required Ψ_m . Zn²⁺ can also be imported into mitochondria via ZnT-type IM transporters, e.g., ZnT2 in mammalian systems.^{17,100–104}

In the study by Malaiyandi et al., targeted mitochondria exhibited a weak fluorescent response, inferring low endogenous levels of labile Zn.¹⁰⁰ Treatment with Zn caused a sudden increase in emission, which indicated an increase in the size of the labile Zn pool. Emission quickly returned to baseline levels, suggesting that mitochondria can rapidly export excess Zn, perhaps via the ZIP8 transporter in mammals.¹⁰⁴ Zn enters mitochondria only when supraphysiological amounts are added. Ψ_m decreases when mitochondria are treated with high levels of Zn, presumably because Zn binds to the exterior of the

organelle.¹⁰⁵ The effect of Zn on the mitochondrial permeability transition pore differs from that of calcium.^{106,107}

High concentrations of mitochondrial Zn, obtained by either incubating isolated mitochondria in solutions spiked with excess Zn or using genetic strains that disrupt Fe metabolism, can have deleterious effects. Fe^{III} nanoparticles accumulate in the mitochondria of yeast cells lacking the frataxin homologue (Yfh1), which also contain deficient amounts of ISCs and hemes.¹⁰⁸ What is less commonly realized is that Zn-protoporphyrin IX accumulates in mitochondria from this same strain.^{109,110} This probably occurs because mitochondrial Fe^{II}, which in WT cells is used by ferrochelatase to metalate protoporphyrin IX, has been converted into oxidized nanoparticles that cannot be installed. Under these conditions, Zn^{II} can substitute for Fe^{II} in the ferrochelatase reaction. The mismetalation of Zn does not arise because of excess Zn^{II}; in fact, yfh1 cells import only a fraction of the Zn imported by WT cells.¹¹¹ Rather, misincorporation arises because of the scarcity of Fe^{II}.¹⁰¹ Accordingly, Zn-protoporphyrin is also observed in Fe-deficient cells.^{101,109,110,112} Curiously, excess ZnSO₄ in the medium (a) prevents the accumulation of Fe in mitochondria of yfh1 cells, (b) increases the growth rate of this strain, and (c) mitigates ROS damage. Surprisingly, these responses are not caused by an increase in ISC or heme synthesis, which makes them difficult to explain. Accumulation of Fe in ISC mutants is thought to arise from insufficient ISC (and/or heme) biosynthesis. Lower-than-normal ISC/heme biosynthetic activities are thought to diminish the rate of export of X-S, the unknown sulfur-containing product that is a cytosolic signaling molecule for these mitochondrial processes. X-S has been posited to regulate cellular Fe import via a signaling pathway involving glutaredoxins and the transcription factors Aft1/2.¹¹³ The fact that Zn added to the exterior of mitochondria suppresses this regulatory mechanism implies that it can functionally substitute for X-S, but how this might work is not obvious. Excess Zn inhibits the TCA cycle, decreases the rate of respiration, inhibits the respiratory electron transport chain,¹¹⁴ and stimulates ROS production.^{98,99} Gazaryan et al. found that picomolar concentrations of aqueous Zn inhibit α -ketoglutarate-dependent mitochondrial respiration by inhibiting lipamide dehydrogenase.¹¹⁵ Perhaps these inhibitory effects indirectly regulate Fe import.

Also involved in mitochondrial Zn metabolism is Mzm1, a soluble 14 kDa protein found in the matrix that helps maintain the labile Zn pool in the matrix and stabilize respiratory complex III.^{96,116} mzm1 mutant cells grow poorly on nonfermentable carbon sources when Zn is limited in the growth medium. Mitochondria isolated from these cells contain decreased amounts of Zn, but not Fe, Cu, Mn, or Mg. They also exhibit decreased respiratory complex III activity. Zn is also critical for autophagy.¹¹⁷ Zn deficiency induces apoptosis by activating the mitochondrial cell death pathway.¹⁰¹

The concentration of labile Zn in mitochondria is reported to be extraordinarily low.⁴ McCranor et al.¹¹⁸ used a FRET-based biosensor derived from carbonic anhydrase II variants that were targeted to the matrix of mitochondria of mammalian cells. The biosensor was exquisitely sensitive to and specific for labile Zn. They reported a labile Zn concentration in the matrix of ~0.15 pM! When cells were deprived of glucose and O₂ for 3 h and then reperfused in O₂, there was an initial surge in labile mitochondrial Zn (as cytosolic levels declined); mitochondrial Zn levels returned to normal after reperfusion for 2

h. In another study, Park et al.¹¹⁹ used a genetically encoded protein-based Zn sensor that was targeted to mitochondria to measure the labile Zn concentration in the organelle. Zn binding induced a conformational change that was monitored by FRET. They reported a concentration of 0.14 pM for labile Zn, far lower than the concentration of the labile Zn pool in the cytosol.

Online LC–ICP-MS analysis of the FTS from soluble mitochondrial extracts revealed a dominant Zn feature corresponding to a mass of 1200 Da (Figure 2).⁴³ Zn₁₂₀₀ was reproducibly present in LC traces of FTSs from fermenting yeast and mammalian mitochondrial extracts. FTSs from mammalian samples contained a second Zn species that migrated according to a mass of ~1500 Da. Zn₁₅₀₀ was not observed in mitochondria from fermenting yeast cells. The concentration of Zn₁₂₀₀ in mitochondria was calculated to be ~110 μ M. This estimate is 9 orders of magnitude greater than the labile Zn concentration reported by McCranor et al.¹¹⁸ and Park et al.¹¹⁹ We are unable to reconcile these inconsistencies. Zn₁₂₀₀ does not appear to be an artifact, as this feature is reproducibly observed, and it migrates in accordance with a mass significantly higher than that of aqueous (“free”) Zn. One possibility is that the fluorescent signals generated by mitochondrially targeted chelator sensors are detecting labile Zn species that are present at concentrations lower than the detection limit of the ICP-MS instrument. Although the ICP-MS instrument is sensitive, it is not sensitive enough to detect a Zn species with a concentration of 0.15 pM. A Zn species at this concentration, contained within a volume the size of mitochondria (~10⁻¹⁵ L), corresponds to just one labile Zn atom per ~10,000 mitochondria. Detecting such incredibly low concentrations would seem to require single-molecule fluorescence methods and a statistical study in which tens of thousands of mitochondria are examined. No such study has been published, raising concern that the reported subpicomolar concentrations of labile Zn in mitochondria severely underestimate the actual concentration.

MANGANESE

MnSod2 is directed to the mitochondrial matrix by an N-terminal targeting sequence.¹²⁰ Once inside the matrix and once the targeting sequence has been clipped, the resulting apoprotein folds with help from Hsp60/Hsp10.^{121,122} In the absence of its targeting sequence, apo-Sod2 is neither folded nor metalated, suggesting that metalation occurs during folding of Sod2.

How cytosolic Mn is transported into the matrix for metalation is unknown. As a member of the MCF, Mtm1 was initially considered to be a Mn transporter and was thus named the manganese trafficking factor for mitochondrial Sod2 because cells lacking it exhibit low Sod2 activity.^{111,123} Contrary to the behavior expected for this role, the concentration of Mn in the matrix was higher in *mtm1* mutants than in WT cells. Attention turned to iron when the absence of Mtm1 was found to cause Fe to accumulate in the matrix. Iron accumulation along with the propensity of bacterial MnSods (e.g., *Escherichia coli* MnSod) to misincorporate Fe seemed to explain the diminished MnSod2-based activity in the *mtm1* yeast strain. The results of initial chromatography studies of Sod2 supported the misincorporation hypothesis.¹²⁴ Namely, when *mtm1* soluble extracts were

chromatographed, most of the eluted Fe comigrated with Sod2. In chromatographs of WT extracts, most of the eluted Mn comigrated with Sod2.

An X-ray absorption spectroscopy study¹²⁵ subsequently demonstrated that the vast majority of Fe that accumulated in *mtm1* mitochondria had not misincorporated into apo-Sod2. A corresponding Mössbauer study revealed that mitochondrial Fe accumulated as Fe^{III} oxyhydroxide nanoparticles,¹²⁶ similar to the Fe accumulation phenotypes observed in ISC mutants such as Yah1-depleted or Atm1-depleted mitochondria.^{127,128} Moreover, little of the Fe that accumulated in *mtm1* mitochondria was in a form (i.e., Fe^{II}) that could be misincorporated. Another surprise was that Fe did not accumulate in *mtm1* cells grown anaerobically, but they did have low Sod2 activity. This indicated that the two phenomena, Fe accumulation and low Sod2 activity, were independent.¹²⁶

We used our LC-ICP-MS system in an effort to detect misincorporated Fe Sod2. In chromatograms of extracts from *mtm1* versus WT cells, we observed reduced protein and activity levels of MnSod2 but no increase in putative FeSod2 features.¹²⁶ Quantitative accounting of Mn concentrations and Sod2 protein levels revealed that the majority of Sod2 protein was missing in soluble fractions, suggesting that apo-Sod2 proteins in mitochondria lacking Mtm1 are less stable toward folding and metalation with Mn.

Supporting this new hypothesis was a LMM Mn species whose concentration was increased in *mtm1* mitochondrial extracts, as would be expected if apo-Sod2 was unfolded and unstable and could not be metalated. The LMM Mn species (called Mn₂₋₃) was estimated to have a molecular mass of 2000–3000 Da. Mn₂₋₃ was also present in WT cells, but at a concentration of just 1 μ M (12% of mitochondrial Mn). In *mtm1* mitochondria, the corresponding concentration was 22 μ M (80% of mitochondrial Mn). In WT mitochondria from cells supplemented with excess MnCl₂, the concentration of Mn₂₋₃ increased to 23 μ M (there was no change in MnSod2 levels). Because MnSod2 was the only Mn-containing peak in chromatograms and Mn₂₋₃ was the only LMM Mn species observed, we hypothesized that Mn₂₋₃ was imported into the matrix through an unknown IM transporter and that this complex was used to metalate apo-Sod2. We further hypothesized that Mtm1 imports a species that is required for the maturation, activity, or stability of apo-Sod2, and that there is a competition of maturation and metalation versus misfolding and degradation. A recent study by Whittaker et al.¹²⁹ reveals that Mtm1 binds pyridoxal 5'-phosphate (PLP) with micromolar affinity, prompting them to conclude that Mtm1 transports PLP into the matrix. Consistent with this, PLP-dependent proteins in *mtm1* mitochondria lack this coenzyme. Heme and ISC biosynthetic pathways depend on PLP, explaining the connection between Mtm1 and mitochondrial Fe metabolism, but how a deficiency in PLP translates into a decline in Sod2 activity in *mtm1* cells remains a mystery; the case would be settled if mitochondria were found to contain a PLP-dependent protein that mediates the folding, maturation, and/or metalation of apo-Sod2.

In our most recent LC-ICP-MS study,⁴³ a similar proportion of mitochondrial Mn (20% of 16 μ M) was due to LMM Mn species. However, chromatograms of both fermenting yeast and mammalian mitochondrial FTSs exhibited slightly different species relative to what was found in our first study (Figure 2). They were both dominated by a Mn complex (Mn₁₁₀₀)

that migrated with a mass of 1100 Da. A second species at 2000 Da (Mn_{2000}) was present in traces from mammalian mitochondria but not from fermenting yeast mitochondria. These results vary from those of our earlier study in which only Mn_{2-3} was evident. We initially suspected some type of calibration error (such that Mn_{2-3} maps to Mn_{1100}), but we have no evidence of this. We also considered that Mn_{2-3} maps to Mn_{2000} ; however, Mn_{2000} has not been observed at significant levels in mitochondria from fermenting yeast, whereas either Mn_{2-3} or Mn_{1100} is routinely observed.

Mn_{2000} may function to deliver Mn to apo-arginase II, a dimanganese enzyme located in the matrix of mammalian mitochondria.^{130,14} This enzyme has an N-terminal presequence typical of matrix enzymes.^{131,132} Arginase II appears to help regulate NO synthesis by competing with nitric oxide synthase for L-arginine. The enzyme may also help regulate hepatic ureagenesis.¹³³ Mammalian cells also contain arginase I, another dimanganese enzyme, but it is cytosolic and expressed mainly in the liver where it functions in the urea cycle.^{134,131} The urea cycle in mammals involves both cytosolic and mitochondrial enzymes. Interestingly, the OM exhibits most of the arginase activity of isolated mitochondria, apparently due to cytosolic arginase I that weakly associates with the OM.^{133,135,136} In yeast, the only isoform of arginase is in the cytosol. However, there is some metabolic association with mitochondria, in that the biosynthesis and breakdown of arginine involve both mitochondrial and cytosolic enzymes.^{137,138} The subcellular location of some enzymes involved in these processes is regulated by the energy status of yeast cells.¹³⁹

COBALT

Cobalt enters mammalian cells in the form of cobalamin, which is then transported to various intracellular locations. The cobalamin delivered to mammalian mitochondria is used to metalate apo-MUT, the only known Co-containing enzyme in this organelle.¹⁴⁰ However, the mitochondrial cobalamin transporter on the IM remains obscure. Once co(II)balamin reaches the matrix, it binds ATP:cob(I)alamin adenosyltransferase (MMAB), is reduced to the Co(I)balamin state, and then accepts a 5-deoxyadenosyl group from ATP.¹⁴¹ MMAB then incorporates the resulting adenosylco(III)balamin into apo-MUT.¹⁴² MMAA, a GTPase “gatekeeping G-protein chaperone”, binds MMAB and apo-MUT to regulate the installation of adenosylcob(III)alamin.¹⁴³ Our LC-ICP-MS traces (Figure 2) reveal one major LMM Co complex with a mass of 1200 Da.⁴³ In mammalian mitochondria, there is a second minor feature at 1500 Da, which may be adenosylcobalamin (1580 Da). Several minor species, possibly cobalamin degradation products, were detected at lower masses, but none of them was reproducibly observed.

MOLYBDENUM

The mechanism by which sulfite oxidase (SO) is assembled in the IMS of mammalian mitochondria and then metalated with the molybdopterin cofactor (Moco) and heme centers has been investigated.¹⁴⁴ Mitochondrial localization is dictated by an N-terminal sequence that targets this protein to the IMS via the TOM complex. Upon being imported into the IMS, the targeting sequence is cleaved by mitochondrial IM peptidase, followed by the

incorporation of Moco into the apo-SO protein, which initiates folding and traps the enzyme within the IMS. SO also contains heme *b*, but this cofactor does not play a major role in folding or trapping. How Moco is shuttled across the OM is unclear; it might pass through a transporter or just diffuse through VDAC pores. The assembly of molybdopterin requires the coordinated action of multiple enzymes in the cytosol,¹⁴⁵ such that Moco is likely installed as a complete unit into mitochondrial Mo enzymes. Moco is unstable in protein-free solutions,¹⁴⁶ suggesting that its translocation into the IMS coincides with its insertion into apo-SO.¹⁴⁴ A pool of free, non-protein-bound Moco was detected in mitochondria, which might be an intermediate in the installation process.

Two Mo-containing enzymes, mARC1 and mARC2, are associated with the OM of mammalian mitochondria where they function with cytochrome *b*₅, NADH, and NADH-cytochrome *b*₅ reductase to reduce various N-hydroxylated compounds and convert nitrite ion into nitric oxide.^{147–149} Both mARC1/2 proteins are small (6–22 kDa) and possess broad substrate specificity. Developmental expression profiles reveal distinct differences between the two isoforms, suggesting that their expression is controlled by independent regulatory mechanisms. mARC2 is expressed in only adult livers where it is involved in lipogenesis and is regulated by nutritional status. This protein may have dual localization in mitochondria and peroxisomes. mARC1 is expressed in both adult and fetal tissues.¹⁵⁰ It has characteristics of a typical OM protein in which the C-terminal catalytic domain is exposed to cytosol and the N-terminal domain points toward the IMS.¹⁵¹ Moco in the SO family of molybdenum hydroxylases contains a Mo center coordinated by a cysteine thiolate and two oxo groups. Moco in mARC1/2 is of the SO type,¹⁵² suggesting that the Moco unit, which is installed in both SO and mARC1/2, originates from a common source. We detected a LMM Mo species with a mass of ~730 Da (Mo₇₃₀) in FTSS from mammalian mitochondria but not from fermenting yeast (Figure 2). Mo₇₃₀ is probably related to Moco (521 Da). We also observed an intense peak near the void volume, indicating high-molecular mass species. Although we cannot determine the exact mass of these species, mARC1/2 enzymes should elute in this region of the chromatogram.

CONCLUSION

Yeast and mammalian mitochondria contain numerous labile LMM metal complexes, as summarized in Figure 3, but consensus regarding some important details is still lacking. Mitochondria contain two or three labile LMM Fe species with estimated concentrations ranging from 5 to 150 μ M (1–20% of total mitochondrial Fe). These Fe complexes are likely imported into the matrix via the mitoferrin1/2 (Mrs3/4) and/or Rim2 proteins. Spectroscopic studies correlate these observed LMM Fe complexes with a mitochondrially localized pool of mononuclear NHHS Fe^{II} species that probably serve as feedstock for the biosynthesis of ISCs and heme centers.

With regard to labile Cu, there are currently two basic scenarios that differ somewhat from each other. One is that ~80% of the Cu in mitochondria is labile and associated with a complex (CuL) whose mass is 13,000 Da. CuL is located primarily in the matrix where it functions as a bioavailable Cu reservoir for the cell. Cytosolic CuL is passed into the matrix via IM transporters Pic2 and Mrs3. CuL in the matrix is shuttled into the IMS, as needed, to

metalate cytochrome *c* oxidase and Cu/Zn Sod1. The other scenario is that ~20% of the Cu in mitochondria is labile and that the labile Cu is a LMM Cu complex whose mass is ~5000 Da and located in the IMS. Cytosolic Cu passes through VDAC porins embedded in the OM to deliver Cu₅₀₀₀ directly into the IMS. Further studies are required to reconcile the differences between these two scenarios.

Mitochondria of cells grown on Zn-sufficient medium contain one labile Zn species with a mass of ~1200 Da (Zn₁₂₀₀). This LMM complex is potentially used to metalate various Zn apoproteins as they fold within the organelle. Additional Zn can be imported into mitochondria (e.g., in cells grown on high-Zn medium or in isolated mitochondria treated with high concentrations of Zn), perhaps due to a loss of membrane potential. However, such high levels of labile Zn are deleterious to mitochondrial function, and in this case, Zn is actively exported from the organelle.

There are one or two labile LMM Mn species present in mitochondria. These could be used to metalate MnSod2 and arginase II (exclusively for mammalian mitochondria). Early studies suggested that apo-Sod2 is mismetalated with Fe in mitochondria lacking Mtm1, a MCF protein. Later studies showed that apo-Sod2 is unstable in the absence of Mtm1 and that Mtm1 transports PLP into mitochondria.

Cobalamins that enter mammalian mitochondria are subsequently incorporated into apo-methyl-malonyl-CoA mutase; however, little is known regarding the transporter(s) involved. Isolated mitochondrial extracts contain a few LMM Co complexes, including some that are probably artifacts or degradation products.

Translocation of the molybdopterin cofactor Moco into the IMS of mammalian mitochondria is followed by its insertion into sulfite oxidase and mARC1/2 enzymes. How Moco passes into the IMS has not been characterized. LMM Mo species are observed in mitochondrial extracts from mammalian samples but not from yeast.

In this review, we have highlighted the current state of this burgeoning field, detailing recent discoveries and current “trials and tribulations.” We hope that doing so will encourage further discussion and inspire new experiments that will resolve these unsettled issues and advance the field. Labile LMM metal complexes can be isolated, so separation methods will dominate future efforts to catalog and structurally characterize them. Chelator-based methods will be used to monitor these same complexes in live, intact cells. As sensors become more specific and sophisticated, they will become more useful in understanding the cellular function of these complexes. Both approaches, working together in conjunction with the power of molecular genetics and bioinorganic spectroscopy, will create a synergy that will ultimately establish the structures and physiological roles of these intriguing little metal complexes in cell biology and medicine.

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ABBREVIATIONS

Afg3	ATPase family gene 3
ATM1	ABC transporter, mitochondrial
ERV1	essential for respiration and viability
FTS (or FTSs)	flow-through solution (or solutions)
HOT13	helper of Tim
ICP-MS	inductively coupled plasma mass spectrometry (or spectrometer)
IM	inner membrane of mitochondria
IMS	intermembrane space
ISC	iron–sulfur cluster
LC	liquid chromatography
LC–ICP-MS	system consisting of a liquid chromatograph (contained in a refrigerated argon atmosphere glovebox) interfaced online to an ICP-MS instrument
LMM	low-molecular-mass
mARC1 and -2	mitochondrial amidoxime reducing components isoforms 1 and 2, respectively
MAS1/2	β and α subunits of the mitochondrial processing protease MMP
MCF	mitochondrial carrier family
MDJ1	mitochondrial DnaJ
MIA40	mitochondrial intermembrane space import and assembly
MMAB	ATP:cob(I)alamin adenosyltransferase
MMAA	GTPase that forms a complex with MMAB and apo-MUT
Moco	molybdopterin cofactor
MUT	methyl-malonyl-CoA mutase
NHHS	non-heme high-spin
OM	outer membrane of mitochondria

Phen	1,10-phenanthroline
PLP	pyridoxal 5'-phosphate
SO	sulfite oxidase
SOD	superoxide dismutase
TOM	translocase of the outer membrane
VDAC	voltage-dependent anion channel

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Figure 1. Design of the LC-ICP-MS experiment.⁴³ Crude mitochondria are pelleted from the soluble fraction of a cell extract and then purified via density gradient ultracentrifugation. Isolated mitochondria are washed, treated with Triton X-100, and centrifuged. The soluble supernatant is passed through a 10 kDa cutoff membrane, and the FTS is injected onto two Superdex peptide size-exclusion columns attached in series. The eluate can be split such that a portion is sent to a fraction collector and the remainder flows to the ICP-MS instrument.

Figure 2. Size-exclusion chromatograms of LMM mitochondrial FTSs detected for Fe, Cu, Zn, Mn, Co, and Mo. Representative traces from our previous study⁴³ highlighting reproducibly observed LMM metal complexes (indicated by a dashed line and a peak label). 10x refers to batches in which the concentration of the indicated metal ion was 10-times normal.

Figure 3. Summary of labile LMM metal complexes found in fermenting yeast and mammalian mitochondria. The size of each block is proportional to the relative concentration of a given species. Suggested submitochondrial locations have not been established.