Filling the mitochondrial copper pool

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A host of critical metalloproteins reside in mitochondria, where metallation occurs within the organelle after protein import. Although the pathways by which proteins are imported into the mitochondria are well known, the mechanisms by which their metal partners are imported are more obscure. A new study by Boulet *et al.* demonstrates that the mammalian SLC25A3 inner membrane transporter, previously known as a phosphate carrier, is also a functional Cu(I) importer, clarifying the source of mitochondrial copper and raising new questions about cellular copper homeostasis.

Metalloenzymes and metalloproteins containing iron, zinc, copper, or manganese ions as cofactors are required in mitochondria to maintain normal physiology. These metalloproteins are synthesized in the cytoplasm and imported into the mitochondria as unfolded polypeptides, with the subsequent metallation pathways occurring within the organelle. The metal-assembly pathways for copper enzymes cytochrome *c* oxidase (COX)[2] and superoxide dismutase-1 (SOD1) rely on bioavailable metal ion pools within the organelle to facilitate metallation, but these pools must be carefully controlled as perturbation through disease or environmental factors may lead to detrimental effects such as mismetallation. However, the mechanisms by which metals are imported into mammalian mitochondria are generally poorly defined. Boulet *et al.* (1) now advance our understanding of copper import in their demonstration that the ortholog of the yeast importer, already known to function as a phosphate carrier, serves a dual function in importing copper as well.

Metal ion transporters that mediate the cellular acquisition of iron, zinc, copper, and manganese are known. However, despite extensive research on mitochondrial metalloprotein targets, a paucity of information exists on mechanisms of metal uptake into this central organelle. Of the mentioned metal ions, only the importers of mitochondrial Fe(II) were known previously. The Fe(II) importers, designated mitoferrins, are part of a family of highly conserved mitochondrial carriers that reside within the inner mitochondrial membrane. *Saccharomyces cerevisiae* contains 35 carrier proteins that transport metabolites into the matrix such as pyruvate, citrate, ADP, and phosphate. The two yeast mitoferrins (Mrs3 and Mrs4) are critical for cell survival in conditions of iron limitation, but not in iron-sufficient cultures (2). Thus, additional Fe(II) transporters exist, one of which may be Rim2 (3). The two mammalian mitoferrins do not exhibit the same redundancy as in yeast (4). Mutagenesis studies identified three conserved histidine residues to be critical for Mrs3 function and its ability to translocate Fe(II), suggesting that ionic Fe(II) is the substrate.

Copper is required within the mitochondrion for the function of two metalloenzymes, COX and SOD1. Copper metallation of these two enzymes occurs within the mitochondrial intermembrane space (IMS) and is mediated by metallochaperone proteins, although the source of the copper is a labile copper-ligand pool localized to the matrix (5). Studies in yeast demonstrated that Pic2, another mitochondrial carrier protein, contributed to copper uptake (6). Analogous to the situation with mitoferrins in yeast where they only show an iron defect in iron-limiting medium, yeast lacking Pic2 exhibited a growth defect on respiratory carbon sources only under copper-limiting conditions. Isolated mitochondria from *pic2*Δ cells contained ~40% of wild-type levels of copper (6), so other transporters were also functional in copper uptake. Mrs3 was shown to have a dual function in the import of both Cu(I) and Fe(II) (7). Yeast lacking both Pic2 and Mrs3 showed a marked copper-dependent growth defect as well as copper-deficient mitochondria.

The Pic2 ortholog in metazoan cells, SLC25A3, is one of 53 mitochondrial metabolite carriers that functions as a phosphate carrier. Mutations in SLC25A3 result in muscle hypotonia, cardiomyopathy, and lactic acidosis in humans (8). Boulet *et al.* (1) demonstrate that murine and human cells depleted of SLC25A3 exhibit a COX deficiency that can be rescued with supplemental copper salts but not phosphate (1). Furthermore, depletion or deletion of SLC25A3 resulted in marked decreases in COX activity and mitochondrial copper levels. Quantitation of the labile matrix copper pool using a mitochondrial-targeted Cu(I) fluorophore revealed that SLC25A3$^{-/-}$ cells had only 25% of wild-type levels of mitochondrial copper. Heterologous expression of human SLC25A3 in yeast lacking Pic2 restored normal mitochondrial copper uptake and rescued the COX defect. Recombinant SLC25A3 reconstituted within liposomes was also shown to transport Cu(I). These studies elegantly demonstrate that SLC25A3 is a mammalian mitochondrial copper transporter and the outcomes of SLC25A3 deficiency in human and murine cells are not merely a secondary effect related to disruption of phosphate transport.

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[2] The abbreviations used are: COX, cytochrome *c* oxidase; SOD1, superoxide dismutase-1; IMS, intermembrane space.

Yeast *pic2*Δ cells do not exhibit any overt phosphate defects. The situation in mammals is more complex because SLC25A3 is a known phosphate transporter. Although it would be unusual for a carrier protein to transport both a cation and anion, it is conceivable that the form of Cu(I) translocated is an anionic copper-ligand complex analogous to the form of Cu(I) within the mitochondrial matrix (9). Alternatively, SCL25A3 may be multifunctional in substrate specificity. A precedent exists in that yeast Rim2 was reported to function in both iron and pyrimidine transport (3). Cells devoid of Rim2 exhibit concordant iron and pyrimidine defects. Since copper-activation of SOD1 and COX occurs within the IMS, an unresolved issue concerns how the matrix labile Cu(I) is translocated back to the IMS for the subsequent activation steps. Mitochondrial carriers are exchangers, so movement could be bidirectional if different Cu(I) ligand complexes exist.

One major unexpected result from the study by Boulet *et al.* 1) was that cells depleted of SLC25A3 exhibited attenuated SOD1 activity and protein levels. Although SOD1 is found in the mitochondria, the bulk of SOD1 is located in the cytoplasm. Copper activation of SOD1 within the IMS occurs within that compartment, since the protein is imported as an unfolded polypeptide prior to its activation. There is no known mechanism for facilitated protein export from this compartment as holoproteins (*i.e.* with bound cofactors); therefore, it is unclear how the availability of matrix copper in SLC25A3-deficient cells could reduce copper-loaded SOD1 in the cytoplasm. However, previous studies have shown mitochondria are critical to cellular copper distribution with mutation of the inner membrane copper protein SCO1/2 affecting cellular copper status (10). More research will undoubtedly shed light on this unexpected crosstalk in cellular copper homeostasis.

The discovery of SLC25A3 as a mammalian mitochondrial copper importer is an impressive accomplishment and should galvanize further research to identify the secondary transporters for copper and iron and the primary transporters for zinc and manganese to have a complete picture of how mitochondria fill and use their metal pools.

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