Divalent metal ions in plant mitochondria and their role in interactions with proteins and oxidative stress induced damage to respiratory function.

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Abstract
Understanding the metal ion content of plant mitochondria and metal ion interactions with the proteome are vital for insights into both normal respiratory function and the process of protein damage during oxidative stress. We have analyzed the metal content of isolated Arabidopsis mitochondria revealing a 26:8:6:1 molar ratio for Fe:Zn:Cu:Mn and trace amounts of Co and Mo. We show selective changes occur in mitochondrial Cu and Fe content following in vivo and in vitro oxidative stresses. Immobilized-metal affinity chromatography (IMAC) charged with Cu$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ was used to identify over one hundred mitochondrial proteins with metal-binding properties. There were strong correlations between the sets of IMAC-interacting proteins, proteins predicted to contain metal-binding motifs and protein sets known to be oxidized or degraded during abiotic stress. Mitochondrial respiratory chain pathways and matrix enzymes varied widely in their susceptibility to metal-induced loss of function, showing the selectivity of the process. A detailed study of oxidized residues and predicted metal-interaction sites in the TCA cycle enzyme, aconitase, identified selective oxidation of residues in the active site and show an approach for broader screening of functionally significant oxidation events in the mitochondria proteome.
Introduction

Transition metal ions are essential in a myriad of biochemical functions by being incorporated into or associate with proteins to elicit functions in living cells. In plant mitochondria, key functions of metal cofactors include metabolism, electron transport, ATP synthesis and the detoxification of reactive oxygen species. For example, Cu and Fe ions facilitate the transfer of electrons in the electron transport chain (Bligny and Douce, 1977; Pascal and Douce, 1993), proteins of the TCA cycle utilize metal ion cofactors to catalyse primary metabolic reactions (Miernyk and Randall, 1987; Jordanov et al., 1992), Mn and Fe are required for antioxidant defense enzymes (Alscher et al., 2002), and Zn is required for the protein import apparatus in both carrier protein transport to the inner membrane (Lister et al., 2002) and in presequence degradation (Moberg et al., 2003). Co is known to substitute for other metal ions in the activation of NAD-malic enzyme and succinyl-CoA ligase from plant mitochondrial extracts (Palmer and Wedding, 1966; Macrae, 1971), but it is not known whether there is an in vivo requirement for trace amounts of Co for plant respiratory metabolism.

Metal ions, however, can also be highly toxic to cells and cell organelle functions. The redox inactive heavy metal Cd exhibits strong affinity for O, N and S atoms (Nieboer and Richardson, 1980) and can inhibit enzyme activity by direct blocking protein function or displacement of natural metal centres. There are numerous reports of heavy metals depleting cellular glutathione pools leading to diminished antioxidant protection in the cell and resulting in ROS accumulation (Schützendubel and Polle, 2002). Cd has been reported to both directly and indirectly inhibit plant mitochondrial function (Kesseler and Brand, 1994; Smiri et al., 2009) as have Co complexes (Guzhova et al., 1979). Redox active metal catalysts such as Cu and Fe cations can also be cytotoxic as they react with ROS via the Haber-Weiss reaction or Fenton-type reactions to produce the hydroxyl anion (OH) (Stohs and Bagchi, 1995). Inhibitory effects of exogenously added Cu and Fe on plant respiratory function have been reported (Kampfenkel et al., 1995; Padua et al., 1996; Padua et al., 1999). Therefore, the presence of free metal cations, both redox active or inactive, in mitochondria may significantly contribute to the initiation and perpetuation of oxidative stress.
One of the best described mechanisms for metal-linked damage is metal-catalysed oxidation (MCO) of proteins which involves the oxidation of susceptible amino acids such as arginine, lysine, proline and histidine among a plethora of other poorly characterized consequences (Stadtman, 1990). It has been proposed that MCO of proteins can be a highly specific event where proteins are more susceptible to damage if they bind metal ions and when the site of protein oxidation can be defined on the protein surface that binds to the metal ions (Stadtman, 1990). One of the major consequences of MCO is the irreversible formation of reactive carbonyls on amino acid sidechains (Stadtman, 1990). Such carbonyls are known to accumulate in the wheat mitochondrial proteome during environmental stress, even more so than in other ROS-producing subcellular organelles of plants (Bartoli et al., 2004). The selectivity of protein susceptibility to MCO was also demonstrated in rice where distinct subpopulations of the mitochondrial matrix proteome were carbonylated following Cu2+ and H2O2 treatment (Kristensen et al., 2004). The targeted damage of select sets of plant mitochondrial proteins has also been observed in other studies, but without clear linkage to the role of metal ions. For example, altered protein abundances has been observed in Arabidopsis (Sweetlove et al., 2002) and pea (Taylor et al., 2005) mitochondria after the initiation of oxidative or environmental stress. Additionally, inhibition of respiratory metabolism by the lipid peroxidation by-product 4-hydroxy-2-nonenal has been shown to operate through modification of a specific subset of proteins (Taylor et al., 2002; Winger et al., 2005; Winger et al., 2007). However, the mechanisms of targeted oxidative modification, the role of metals, and the consequences for mitochondrial metabolic function are not very well understood. Furthermore, whether or not selectivity of protein damage in mitochondria is based on relative metal ion affinity and if the sites of damage can be predicted by the sites of metal ion binding is not known.

In the current study, we investigated metal homeostasis in the Arabidopsis mitochondrion during oxidative stress. The interactions between metal ions and proteins were also investigated using immobilized-metal affinity chromatography (IMAC). Functional assays were used to determine the targets and consequences of metal ion interaction in the mitochondrion and to explore the linkages to the redox nature of the metal and the loss of mitochondrial functions. Finally, a detailed study of the oxidized peptides of aconitase was undertaken to probe the linkage between metal
binding sites, the oxidation of amino acids, and the inactivation of this critical TCA cycle enzyme.
Results

The plant mitochondrial metallome and its changes during oxidative stress

The metal content of mitochondria isolated from Arabidopsis cell culture was analyzed by inductively-coupled plasma mass spectrometry (ICP-MS). This showed Fe, Cu, Zn and Mn to be the predominant species of transition metals in Arabidopsis mitochondria, with trace levels of Co and Mo also detected (Table IA). Fractionating mitochondria into soluble and membrane components and further isolation of an integral membrane portion, revealed the integral membrane proteome contained 3-fold more Cu and Fe than the soluble proteome, on a protein basis (Table IB). This is likely to be due to the abundance of Cu- and Fe-containing electron transport chain components and their enrichment in the integral membrane fraction. Mn was evenly distributed between the soluble and the integral membrane compartment (Table IB).

The redox cycling metals, Cu and Fe, accounted for approximately 75% of the mitochondrial metallome (Table IA). As labile metals are a likely cause of oxidative damage to proteins, ICP-MS was then used to quantify changes in metal composition of mitochondria isolated from cells under oxidative stress. The chemical treatments of cells and timing of analyses were selected based on previous studies of plant mitochondrial oxidative damage in which the products of lipid peroxidation were measured to peak at 8 hours post-treatment (Winger et al., 2005).

Antimycin A is an inhibitor of Complex III that ultimately leads to superoxide production within mitochondria (Maxwell et al., 1999), while menadione treatment of cells is reported to induce a broad cellular superoxide production from membranes (Hollensworth et al., 2000). Treatment of cells with either chemical was compared to a methanol as well as an untreated control to ensure the carrier solvent was not responsible for the changes observed. Both chemicals induced a 1.5-2-fold accumulation of Fe in mitochondria on a protein basis (Table IIA). This accumulation of Fe in mitochondria isolated from stress treated cells could not be traced to either the soluble protein fraction or the integral membrane protein fraction from these mitochondrial extracts (Table IIA), suggesting it was probably in the peripheral membrane fraction that was stripped from the membranes by carbonate extraction prior to ICP-MS analysis. Menadione treatment also elicited a slight reduction in mitochondrial integral membrane Cu content (Table IIA), suggesting damage to membrane-embedded cupro-proteins. The accumulation of Fe following both
menadione and antimycin A treatments may suggest a common mechanism induced by superoxide, regardless of the cellular source.

Damage to metalloproteins from oxidative treatments of cells was most apparent following \( \text{H}_2\text{O}_2 \) treatment. In isolated mitochondria, \( \text{H}_2\text{O}_2 \) induced a 40% decrease in Cu content (Table IIA). This loss of Cu could be traced to both the soluble and integral membrane protein fractions (Table IIA), which implies damage to mitochondrial soluble cupro-proteins and also to cupro-components of the membrane bound electron transport chain. A 40-50% reduction of Fe and Mn content was also observed in the soluble protein fraction of mitochondria from \( \text{H}_2\text{O}_2 \) treated cells (Table IIA). This suggested that matrix metalloproteins such as Fe-S containing aconitase and Mn-containing SOD are sensitive to damage by \( \text{H}_2\text{O}_2 \). The observed metal losses are consistent with the fact that hydrogen peroxide is known to be able to damage proteins by fragmentation, destroying metal coordination sites (Hunt et al., 1988) and by evidence that plant aconitase is easily inactivated by \( \text{H}_2\text{O}_2 \) (Verniquet et al., 1991).

To provide further evidence of direct oxidative damage to metalloproteins causing loss of metal ions, mitochondria isolated from untreated cells were directly treated with \( \text{H}_2\text{O}_2 \) \textit{in vitro} and the metal content of whole mitochondria and across different mitochondrial compartments was measured by ICP-MS (Table IIB). In total mitochondria samples, a significant reduction in Fe content was observed, and this loss was localized to the soluble fraction and not the integral membrane protein fraction. This apparently release of Fe from the soluble compartment could not be detected in low molecular mass fractions obtained by size exclusion chromatography of mitochondrial extracts but this is probably due to the detection limitations of the ICP-MS (data not shown).

**Inhibition of respiratory function by labile metal cations**

Transition metal ions generally do not exist as free cations in cells but are sequestered by organic acids or protein ligands to minimise damage resulting from redox cycling with reactive oxygen species (Rauser, 1999). However, in the current study, ICP-MS has provided evidence that metal homeostasis can be disrupted during oxidative stress. During this time of increased metal flux into and out of the mitochondria, it is likely that the metal ions exist in a labile state and hence bind to
and could have toxic effects on other proteins. Historical studies have noted that exogenous addition of metal ions affect respiratory complexes and consequently mitochondrial energy production (Skulachev et al., 1967; Kesseler and Brand, 1994; Padua et al., 1996; Kuznetsova et al., 2005). Hence, the respiratory capacity of mitochondria was measured following addition of Cu$^{2+}$, a redox active ion, or Zn$^{2+}$, a redox inert metal ion, to characterise the differential effects of free metal ions on mitochondrial respiratory function.

A dose response procedure was undertaken using treatments for 5 mins with metal ions at 10, 50 and 100 $\mu$M concentrations compared to untreated control samples. This gave metal ion:protein ratios of 83, 415 and 830 nmol metal ions per mg of protein, respectively. At 10 $\mu$M metal ions, no detrimental effects on respiration could be observed. However, at 50 $\mu$M and 100 $\mu$M, metal ions inhibit mitochondrial respiratory rate (Table III). The electron transport chain (ETC) and tricarboxylic acid (TCA) cycle substrates used were succinate, NADH, and glutamate+malate. Each of these substrates initiates mitochondrial respiration via a different pathway: succinate-dependent respiration measures the activity of the succinate dehydrogenase complex and the subsequent ETC; NADH-dependent respiration measured external NADH dehydrogenase entry to the ETC, bypassing Complex I; whilst the organic acids glutamate+malate are substrates for the TCA cycle which ultimately produces NADH in the matrix that enters the ETC at Complex I and/or the internal alternative NADH dehydrogenases.

Succinate-dependent respiration was susceptible to Cu$^{2+}$ and Zn$^{2+}$ treatment resulting in at least a 40% decline in respiration at both 50 $\mu$M and 100 $\mu$M of each metal ion (Table IIIA). In contrast, external NADH-dependent respiration was diminished differentially by Cu$^{2+}$ and Zn$^{2+}$. The toxicity of Cu$^{2+}$ toward NADH-dependent respiration was more pronounced as almost 80% inhibition of respiration was observed at both 50 $\mu$M and 100 $\mu$M Cu$^{2+}$ concentrations. On the other hand, Zn$^{2+}$ could only induce 40% inhibition of respiration under the same metal ion concentrations examined. Cu$^{2+}$ and Zn$^{2+}$ did not significantly affect glutamate/malate-dependent respiration.

To determine if the cytochrome or alternative pathway were differentially affected by metal ion treatment, inhibitors were used to analyse each one independently. Cytochrome pathway inhibition by metal ions could be observed at 50
and 100 μM concentrations but did not occur in a dose-dependent manner as the extent of the inhibition appeared to plateau (Table IIIB). A 40-60% inhibition of respiration via the cytochrome pathway with either succinate or NADH as a substrate was observed following addition of either metal ion. Zn^{2+} but not Cu^{2+} was found to significantly inhibit the glutamate/malate-driven cytochrome pathway respiration. The alternative oxidase pathway was inhibited sharply by Cu^{2+} at 50 μM and this metal ion could almost completely abolish respiration at 100 μM during both succinate and NADH driven alternative oxidase-dependent respiration. Zn^{2+} was also able to inhibit alternative pathway respiration but to a lesser extent (Table IIIC).

Selective interaction of mitochondrial proteins with immobilised metal cations

The interaction of metal ions with mitochondrial proteins that might be responsible for these toxic effects were investigated in vitro using immobilised metal affinity chromatography (IMAC) to trap metal-binding proteins. Initial studies used Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} or Zn^{2+} to charge the IMAC resin (Figure 1A). Arabidopsis mitochondrial proteins solubilised in 0.1% (v/v) Triton X-100 were introduced into IMAC columns charged with metal ions of interest. Proteins that were metal-binding were retained on the IMAC resin whilst unbound proteins were removed in wash steps. The bound proteins were eluted from the IMAC resin by stripping the metal ions with EDTA. The eluent was concentrated, desalted and analysed by SDS-PAGE and then compared to total mitochondrial protein extracts to determine the specificity of protein enrichment. This showed that only Co^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+} retained subsets of mitochondrial proteins (Figure 1A). These metal ions were then used to explore the differential interactions of divalent metal ions with proteins and how these interactions potentially modulate protein function. To further eliminate apparent non-specific binding of proteins to IMAC, fractionation of the proteins into weakly and strongly interacting sets by electrostatic or competitive displacement, with NH_{4}Cl or imidazole respectively, was conducted. The enriched bands were excised for protein identification by mass spectrometry (MS). For MS protein identification of SDS-PAGE bands, multiple proteins are often identified and only the major proteins identified from each band were reported (Tables S1-S4).
**Cu^{2+}-interaction proteins** - Initial time course studies of proteins binding to Cu^{2+}-IMAC revealed that proteins bound rapidly and 1 min incubations were sufficient to permit binding (data not shown). However, the protein profile of bound proteins was very complex and showed poor selectivity compared to whole mitochondrial samples (Figure 1A). Various concentrations of imidazole were investigated but resulted in poor fractionation of proteins based on strength of binding (results not shown). Ammonium chloride step gradients allowed electrostatic displacement of the Cu^{2+}-bound proteins and improved protein fractionation providing distinct protein profiles based on the strength of protein affinity to Cu^{2+}-IMAC (Figure 1B). Thirty-five proteins where identified in bands from fractions between 0.1 M and 0.6 M NH_{4}Cl and were designated weakly associating proteins (Supp Figure 1 and Table S1) whilst 48 strong Cu^{2+}-IMAC interacting proteins were identified from 0.8 M to 1 M NH_{4}Cl fractions and also from the EDTA stripped IMAC resin (Supp Figure 1 and Table S2). Some of the proteins identified were also found by the Kung et al (2006) study of Cu-binding proteins from whole Arabidopsis root extracts, notably glutathione-S transferases (At2g30860 and At2g30870), ATP synthase β subunit (At5g08670) and aconitase (At4g26870). Additionally, 25 proteins that have not previously been identified by mass spectrometry from plant mitochondria were enriched by Cu^{2+}-IMAC and identified. These previously uncharacterised proteins are indicated in Tables S1,S2. The experimentally derived Cu-interacting protein subset was very different to bioinformatic predictions which have estimated that 0.5% (~150 proteins) of the Arabidopsis proteome consist of Cu-binding proteins with the largest number belonging to the Cu-dependent oxidoreductase class (Andreini et al., 2008). Instead, Cu-interacting proteins were often involved in redox reactions, such as cytochrome c (At1g22840 and At4g10040), electron transport flavoprotein (At1g50940), and a plethora of non-Cu dependent dehydrogenases and reductases (Table S1,S2). Interestingly, the list of Cu^{2+} binding proteins identified here is remarkably similar to the reported list of rice matrix proteins that are carbonylated after Cu^{2+}-induced oxidation of rice mitochondrial extracts (Kristensen et al., 2004).

**Co^{2+}-interaction proteins** - Initially, unfractionated (UF) proteins which were bound to Co^{2+}-IMAC were analysed by SDS-PAGE and revealed an enrichment of some protein bands when compared to the total mitochondrial protein sample (Figure 1A,C).
The 10 bands that were enriched in the unfractionated sample were excised and identified (Figure S2 and Table S3). The separation of weak and strong Co$^{2+}$-interacting proteins was attempted using both competitive and electrostatic displacement of proteins. Imidazole fractionation of Co$^{2+}$-IMAC bound proteins showed most eluted at concentrations of 10-20 mM. Five extra protein bands were enriched in the 20 mM imidazole fraction and excised for protein identification (Figure 1C, Figure S2 and Table S3). Fractionation via electrostatic displacement was also conducted. The majority of Co$^{2+}$-binding proteins could be displaced from the resin using 0.1 M NH$_4$Cl showing the binding of proteins to Co$^{2+}$-IMAC is significantly weaker than that of Cu$^{2+}$-IMAC binding proteins (data not shown). Using Co$^{2+}$-IMAC, 45 proteins involved in detoxification, DNA synthesis, protein fate, protein synthesis, signal transduction and unknown functions were identified. However, energy production and metabolism proteins were by far the best represented functional category (Table S3). Co$^{2+}$-IMAC was able to purify proteins that were found using both Cu$^{2+}$ and Zn$^{2+}$ but the binding of proteins to Co$^{2+}$ appears more similar to Zn as the proteins NADH dehydrogenase subunit 9, cytochrome c oxidase subunit 5b, dihydrolipoamide dehydrogenase, malic enzyme, 2-oxoglutarate dehydrogenase and nucleoside diphosphate kinase were found in common between the Co$^{2+}$ and Zn$^{2+}$ sets but were not in the Cu$^{2+}$ set. Co$^{2+}$-IMAC was also able to purify 19 proteins that could not be purified by the other divalent cations (Figure 1E). Examples of proteins exclusively purified by Co$^{2+}$-IMAC include DAG proteins, subunits of Complex I, methylcrotonyl CoA carboxylase alpha subunit, arginase, and 4 other metabolic enzymes. Co$^{2+}$-IMAC was also shown to be an effective tool in enriching low abundant proteins as 9 proteins previously uncharacterised by MS to be in mitochondria were identified (Table S3).

Zn$^{2+}$-interaction proteins - Unfractionated (UF) proteins which were bound and eluted from Zn$^{2+}$-IMAC were analysed by SDS-PAGE which revealed enrichment of specific proteins bands when compared to the total mitochondrial protein sample (Figure 1A). The 11 bands that were enriched in the unfractionated fraction were excised for identification (Figure S3 and Table S4). The separation of weak and strong Zn$^{2+}$-interacting proteins was also attempted using both competitive and electrostatic displacement of proteins. As with Cu$^{2+}$- and Co$^{2+}$-IMAC, imidazole was
not able to efficiently fractionate proteins bound to Zn\textsuperscript{2+}-IMAC (Figure 1D). Despite poor fractionation of all the IMAC protein sets with imidazole, relative protein binding strength could be determined based on the concentration of imidazole needed to elute a large portion of the proteins. Proteins bound to Zn\textsuperscript{2+}-IMAC more tightly than Co\textsuperscript{2+}-IMAC as the majority of proteins were displaced at 50 mM imidazole in Zn\textsuperscript{2+}-IMAC compared to 10-20 mM imidazole in Co\textsuperscript{2+}-IMAC (Figure 1C and 1D). Electrostatic displacement of Zn\textsuperscript{2+}-interacting proteins using ammonium chloride also verified stronger protein binding to Zn\textsuperscript{2+} compared to Co\textsuperscript{2+} as proteins tended to eluted at 0.7 M NH\textsubscript{4}Cl compared to 0.1 M NH\textsubscript{4}Cl in Co\textsuperscript{2+}-IMAC (data not shown). Eleven additional protein bands observed to have eluted from Zn\textsuperscript{2+}-IMAC in the 50 mM imidazole fraction were also excised and identified (Figure S3 and Table S4). Zn\textsuperscript{2+}-IMAC appeared to have no success in the enrichment of the well-characterised Zn-dependent proteins such as TIMs (Lister et al., 2002) or the metalloprotease PreP (Moberg et al., 2003). Instead, the 74 proteins identified are likely to associate with Zn through a variety of mechanisms. Some examples of these include proteins that contain Zn fingers (cytochrome c oxidase 5b) (Kubo et al., 2006), use Zn as a cofactor (nucleoside diphosphate kinase, mitochondrial processing peptidase beta) (Parks and Agarwal, 1973; Luciano and Geli, 1996), are inhibited by Zn (methylcrotonyl-CoA carboxylase, 2-oxoglutarate dehydrogenase) (Diez et al., 1994; Brown et al., 2000), or interact with divalent cations (malic enzyme, glutamate dehydrogenase, succinate dehydrogenase Fe-sulphur subunits) (Massarini and Cazzulo, 1975; Kindt et al., 1980). Proteins with no known association with Zn ions such as the ATP synthase subunits were also found. Interestingly, Zn\textsuperscript{2+}-IMAC was able to purify 11 subunits of the ATP synthase complex which was greater than any of the other metal-IMAC resins used.

\textit{Fe\textsuperscript{3+}-interaction proteins} – Enrichment of proteins with Fe\textsuperscript{3+}-IMAC was very variable. The major protein enriched in Figure 1A was identified as malate dehydrogenase (At1g53240), but this pattern and specificity can be changed and greatly dependent on the \textit{pH} and ionic strength of the media used. Our data indicated we were largely looking at a pseudocation exchange effect which was in agreement with the findings by (Zachariou and Hearn, 1996) (see Figure S4 for more details).
Metal-binding motifs in mitochondrial IMAC-interacting protein sets - Metals often bind proteins at specific coordination sites involving cysteine, histidine and methionine residues (Harding, 2004). Hence an analysis of the sequences of the proteins in our IMAC subsets was performed to determine if putative metal-binding motifs were more common that expected by random chance in these proteins. For simplicity, the metal-binding motif parameters used were the same as those used by Kung et al (2006) to validate their metal-binding proteins. Kung et al (2006) found that the top 6 statistically enriched motifs in their protein subsets could accounted for nearly 90% of the proteins identified. Kung et al (2006) was also able to show Cu-binding to synthetic peptides carrying a range of these putative Cu-binding motifs but did not characterise the biological significance of such motifs. In our data, 10, 45, and 21 Cys-His-Met motifs were found to be significantly greater in frequency in the Cu, Co and Zn subsets respectively when compared to the entire Arabidopsis proteome or the known Arabidopsis mitochondrial proteome (Tables S5a-i). Figure 2 shows the top 6 enriched motifs for each of the divalent metal cation bind sets. In total 72% of proteins from our Cu-binding subset contained one or more of the top 6 Cu-binding motifs compared to 96% and 89% for Co and Zn respectively (Figure S5), which is comparable to the findings of Kung et al (2006) using this same method. One of the top 6 motifs, ‘H-(X)5-H’, was common to all three datasets and also to Kung et al (2006), while ‘HM’ was shared between our Co²⁺ and Zn²⁺ datasets. His residues were significantly more enriched in the Co-binding protein subset, suggesting that His motifs may complex Co more readily than Cu or Zn.

Relationship between metal ion interaction and modulation of protein function

A range of major TCA cycle enzymes, that were identified to interact in IMAC here and have been reported to be carbonylated in the literature, were selected for activity measurements. The aim was to determine if there was a clear relationship between observed metal ion interactions observed using IMAC, reports of protein oxidation, and modulation of enzyme activity.

Aconitase was found to interact with Cu²⁺ (Table S2) and is reported to be oxidatively modified by Cu-catalysed mechanisms (Kristensen et al., 2004). Aconitase was inhibited 25% by H₂O₂ treatment (Table IV) and this is in agreement with previous studies in plants and mammals (Verniquet et al., 1991; Brazzolotto et
Cu$^{2+}$ was able to diminish aconitase activity by 80% and the primary mechanism of this inhibition appeared to be independent of reducing or oxidising agents (Table IV). Fe$^{3+}$ and Zn$^{2+}$ did not affect aconitase activity directly or by MCO. Interestingly, the presence of Fe$^{3+}$ or Zn$^{2+}$ in the H$_2$O$_2$ treatment was in fact able to protect aconitase activity (Table IV). This may be due to the metal catalysts degrading H$_2$O$_2$ to products that are less toxic to aconitase. The addition of labile Fe$^{3+}$ to the mitochondria may have been beneficial to aconitase activity through restoring of the FeS cluster, damaged by H$_2$O$_2$, from the inactive [3Fe4S]$^+$ state to the active [4Fe4S]$^{2+}$ state (Brazzolotto et al., 1999).

The activity of pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex showed similar patterns of inhibition by metal ions. Both activities were significantly affected by Cu$^{2+}$ and Zn$^{2+}$ by direct mechanisms and were found to be resistant to MCO under the conditions examined (Table IV). The observed toxicity of Cu$^{2+}$ towards pyruvate and α-ketoglutarate dehydrogenase complex activities agrees with in vivo and in vitro studies in mammalian mitochondria (Sheline and Choi, 2004). The toxicity of Zn$^{2+}$ towards α-ketoglutarate dehydrogenase complex activity could at least in part explain the inhibition of glutamate/malate driven cytochrome oxidase-dependent respiration by Zn$^{2+}$ (Table III). Subunits of pyruvate dehydrogenase complex was found by IMAC to interact with Co$^{2+}$ and Cu$^{2+}$ and subunits of α-ketoglutarate dehydrogenase complex to interact with Co$^{2+}$ and Zn$^{2+}$, indicating mechanisms for the direct effect of metal ions on these enzymes.

Fumarase was not found as a metal-interacting protein in our IMAC studies but has been reported to be carbonyl-modified in rice mitochondria by Cu$^{2+}$-catalysed oxidation (Kristensen et al., 2004). In our hands the activity of this enzyme was not affected by MCO (Table IV). However it was the only enzyme investigated that showed sensitivity to ferric ions which caused a 30% decrease in activity (Table IV).

The TCA cycle enzymes isocitrate dehydrogenase and malate dehydrogenase were also found to be putatively oxidised in rice mitochondria (Kristensen et al., 2004). As with fumarase, enzyme assays of Arabidopsis mitochondria showed both isocitrate dehydrogenase and malate dehydrogenase activity to be resistant to H$_2$O$_2$ and MCO under the conditions investigated (Table IV). None of the metal ions used were able to modulate either isocitrate dehydrogenase or malate dehydrogenase
activity despite experimental data from IMAC suggesting interactions with Zn$^{2+}$ and Cu$^{2+}$/Fe$^{3+}$ for each enzyme respectively.

Hence, while a number of TCA cycle enzymes that were selected for analysis showed a functional impact of incubation with metal ions, there was a complex relationship with the metal interaction data (Table IV). As MCO of proteins may not necessarily occur at the active site, protein carbonylation may not be noticeably detrimental in protein function assays. The specificity of carbonylation was further investigated to assess the susceptibility of putative metal-binding sites to oxidation using aconitase as a case study.

**Linking metal binding sites and oxidized residues to protein structure of aconitase.**

Aconitase interacts with Cu$^{2+}$-IMAC (Figure 1B, Figure S1 and Table S2), contains all but one of the top 6 putative Cu$^{2+}$ binding motifs, has reduced activity on Cu$^{2+}$/H$_2$O$_2$ exposure (Table 4) and is reported to be oxidatively modified by Cu-catalysed mechanisms (Kristensen et al., 2004). The 3D structure of the Arabidopsis mitochondrial aconitases (At2g05710 and At4g26970) can also be predicted by using the crystal structure of the human cytosolic aconitase, PDB accession 2b3y (Dupuy et al., 2006), which shares ~60% amino acid identity to the Arabidopsis mitochondrial aconitase proteins.

The location of the putative Cu-binding motifs H-X$_5$-H, M-X$_3$-H, C-X$_7$-H, H-X$_1$-C, M-X$_7$-H and H-X$_2$-M were mapped onto the aconitase structure using a BLAST alignment of the protein sequences (Figure S6). Based on the 2b3y crystal structure, the 2 coordinating His residues of the H-X$_5$-H motif are 18.93 Å and are not likely to form a metal-coordinating pocket. The M-X$_3$-H motif and the conserved C-X$_7$-H, and H-X$_1$-C motifs are clustered together on the protein surface indicating that this region is likely to attract Cu ions, however the distances between the respective coordinating residues are 6.51 Å, 11.58 Å, and 5.25 Å for M-X$_3$-H, C-X$_7$-H, and H-X$_1$-C respectively, which is relatively large as the criteria for metal-coordination normally requires the electron donor to be within ~0.75 Å of the metal ion (Harding, 2004). H-X$_2$-M was the only motif found within the core of the protein, near the aconitase catalytic and substrate recognition site. The predicted distance between the coordinating residues of this motif is 5.75 Å. Whether this site is responsible for Cu
inactivation of aconitase activity remains to be investigated. However, it is unlikely the motif is responsible for aconitase binding to the Cu\(^{2+}\)-IMAC resin as the site is buried within the protein core and is likely to have restricted access to the immobilised Cu\(^{2+}\).

To test whether sites near putative metal-interacting motifs had an enhanced susceptibility to MCO, the soluble protein fraction from Arabidopsis mitochondria was treated with 100 \(\mu\)M Cu\(^{2+}\) and H\(_2\)O\(_2\) to elicit MCO. The band corresponding to aconitase on SDS-PAGE was excised trypsin digested and peptides analysed by mass spectrometry. The variable modifications of oxidation of the amino acids C, D, F, K, M, N, P, R, and Y were used to determine if MCO elicited any detectable changes between mock and treated extracts. In triplicate experiments there were 11 tryptic peptides from aconitase consistently found in both mock and Cu\(^{2+}\) treated samples, in addition three peptides from aconitase were consistently found only in the mock samples and one multiply oxidised peptide was found consistently and only in the Cu\(^{2+}\) treated samples (Figure 3A). This latter peptide is in a highly conserved region of aconitase and includes an Asp and His residue of the active site (Figure 3B, C). A range of other oxidised peptides of aconitase from the oxidised samples were found in single experiments but were not able to be repeatedly observed. These apparently random events typically mapped to the surface of the 3D structure, but are generally not in close proximity to the metal-binding motifs (Table S6, Figure S6).

**Discussion**

Metals are important cofactors in many biological reactions but to date there has been little systematic analysis of the metal composition of subcellular organelles in plants. Our screen of the metallome of Arabidopsis cell culture mitochondria is to our knowledge the first multi-elemental profiling of a subcellular organelle in plants. Fractionation of mitochondrial samples revealed the integral membrane fraction had a 6-fold greater Cu and Fe content than the soluble protein compartment on a protein basis, consistent with the redox transition metals involved in the electron transport chain. The Arabidopsis mitochondrial Fe and Mn content of 3.2 and 0.12 nmol per milligram of protein, respectively, is relatively similar to that of yeast mitochondria which was found to be 5-10 and 0.16-0.36 nmol per milligram of protein, respectively (Luk and Culotta, 2001; Luk et al., 2003; Luk et al., 2005; Yang et al.,
The discovery of trace amounts of Co$^{2+}$ and Mo$^{2+}$ in Arabidopsis mitochondria was somewhat unexpected, however, reports of Co$^{2+}$ substituting for other metals in metal-dependent enzyme reactions in plant mitochondria (Palmer and Wedding, 1966; Macrae and Moorhouse, 1970; Macrae, 1971) and of a mitochondrial Mo$^{2+}$ carrier protein in Arabidopsis (Baxter et al., 2008) are consistent with these data.

Little is known regarding the subcellular perturbations in the metal content during oxidative stress despite the general acceptance that MCO is a common source of oxidative modification in biological macromolecules (Stohs and Bagchi, 1995). Comparing the impact of oxidative stress on the metallome of the Arabidopsis mitochondria using the same chemical elicitors reported by Sweetlove et al (2002) and Winger et al (2005, 2007) allowed the changes in respiratory activity, lipid peroxidation and protein degradation reported in these studies to be considered in light of metal-catalysed reactions investigated here. H$_2$O$_2$ treatment resulted in a detectable loss of Cu from total mitochondria (Table II). Upon fractionation of the mitochondria, a decrease in membrane bound Cu levels was observed suggesting damage to Cu-containing enzymes such as cytochrome c oxidase. There was also a loss of Cu, Fe and Mn observed in the soluble compartment suggesting damage to matrix metalloproteins following H$_2$O$_2$ treatment. Sweetlove et al (2002) observed decreased respiratory rate after H$_2$O$_2$ additions and breakdown of a series of proteins including Fe-containing aconitase, Complex I and superoxide dismutase, as well as the breakdown of a set of matrix enzymes that are here shown to bind to IMAC resins (Tables S1-S4). Menadione treatment, like H$_2$O$_2$, was able to induce loss of Cu in the membrane fraction (Table II) indicating possible damage to the cytochrome c oxidase complex. The proteome changes induced by both these chemical stresses were similar according to Sweetlove et al (2002).

Antimycin A and menadione both appeared to induce a 2-fold accumulation of Fe in mitochondria (Table II). Transcriptional changes in Fe metabolism during oxidative stress suggests that induction of ferroproteins such as cytochrome c and alternative oxidase may account at least in part for these Fe changes following antimycin A treatment (Yu et al., 2001). Winger et al (2007) has reported similar patterns of HNE-modified proteins following antimycin A and menadione treatments, and lipid peroxidation leading to HNE is known to be an Fe$^{2+}$-stimulated process. The disturbance of Fe and Cu homeostasis by menadione has also been supported in a
study of hamster fibroblasts in which menadione treatment increased the lability of both these redox active transition metals (Calderaro et al., 1993).

This current study is also in agreement with wider proteomic studies investigating the changes in protein abundance in Arabidopsis during abiotic stress. Aconitase is known to release ferrous ions when oxidatively damaged (Verniquet et al., 1991; Brazzolotto et al., 1999) and has been reported to decrease in abundance in cadmium-stressed (Sarry et al., 2006), salt-stressed (Jiang et al., 2007) and salt and osmotic-stressed (Ndima et al., 2005) plants. Another mitochondrial metalloprotein that has been shown to have altered abundance following oxidative stress is the Zn-containing mitochondrial processing peptidase (MPP) (At3g02090.1) which increased in abundance in salt and osmotic stress (Ndima et al., 2005). The increase in abundance of MPP may contribute to the import of new proteins to replace degraded proteins. In contrast, while we have not seen any evidence of increases in Mn content in mitochondria, MnSOD has been observed to increase in abundance in salt-stressed (Jiang et al., 2007) and in salt and osmotic stressed Arabidopsis plants (Ndima et al., 2005).

While the release of metal ions from metalloproteins and the import of new metal cofactors into the mitochondrion to synthesise or repair metalloproteins are likely to contribute to the changes in the mitochondrial metallome, they also raise the need to study the way in which metals interact with the proteome as a whole. Examining this metal-protein interactome is extremely complicated and fraught with technical changes. Here we have used IMAC in an attempt to mimic protein binding to free metal ions in vivo. Under the native conditions explored, only exposed metal-binding surfaces are studied and there is no assessment of metal-coordinating sites that are buried in the protein core (Ueda et al., 2003). In addition, steric hindrance caused by the use of immobilised metal ions on a fixed ligand will limit access to metal ion binding sites in some proteins (Ueda et al., 2003). Additionally, the strength of metal coordination in a native metalloprotein may be too strong to allow for an exchange of metal ion cofactors with the IMAC resin. Like the IMAC study of Kung et al (2006), we have found that IMAC selects subsets of proteins that contain significant numbers of putative metal interaction motifs on their surfaces. In the metal-protein interaction studies of Cu ions and liver disease (She et al., 2003; Smith et al., 2004), nickel hypersensitivity in human B cells (Heiss et al., 2005), and Cu ion
homeostasis in Arabidopsis roots (Kung et al., 2006) no attempt was made to localise the metal-binding motifs on homologous proteins. However, the surface localisation of the putative metal-binding motifs is supported by reports of the use of IMAC to assist in protein crystallisation through surface histidine residues (Frey et al., 1996), and reports of protein contaminants in metal affinity purification of His-tagged recombinant proteins due to natural surface metal-binding motifs (Cai et al., 2004; Bolanos-Garcia and Davies, 2006). While the functional significance of these putative surface metal-interacting sites remains undefined, we noted that there was a good correlation between these proteins and experimentally observed sets of proteins that are carbonyl-tagged (Kristensen et al. 2006), lipid peroxide-tagged (Winger et al. 2007) and/or degraded (Sweelove et al. 2002) during oxidative stresses. We thus hypothesised that they might have had an increased susceptibility to metal-catalysed oxidation due to their affinity for metal ions.

Detailed analysis of the activity of major matrix-located enzymes following exposure to oxidative conditions revealed very different responses (Table IV) indicating that the impact of oxidation on function is highly variable, dependent on different sets of conditions and different metal ions and thus specific studies will be required to assess the impact of chemical stresses on each protein (Table IV). In support of this, studies in castor bean peroxisomal proteins have also demonstrated a lack of correlation between the extent of protein oxidation and inhibition of protein function following metal-catalysed oxidation (Nguyen and Donaldson, 2005). The prospect of having to perform functional analysis on all oxidised proteins to infer effects is daunting. However, our study of aconitase revealed that MS analysis of the peptides from damaged proteins and layering onto 3D structures (Figure 3, Table S6) can provide important information. For example, by revealing putative active site damage consistent with the loss of function of this enzyme during oxidative conditions as well as a range of surface localised oxidation sites.

The general proposition that electron-donor dense regions on protein surfaces coordinate metal ions and, in the presence of ROS, the specific metal-coordinating residue(s) are oxidised (Stadtman, 1993), is supported by a series of specific studies. For glutamine synthetase, it was observed that in a peptide containing a stretch of oxidation-prone amino acids, Met268-His269-Cys270-His271-Met272, only the metal-coordinating His269 was oxidised (Farber and Levine, 1986). The theory of ‘caged
MCO’ has been applied to the characterisation of Cu-binding residues of the cuproproteins Cu/ZnSOD and azurin (Bridgewater et al., 2006) and the known metal-interacting proteins angiotensin I and bacitracin (Bridgewater et al., 2006, 2006). The sites of oxidation which were found to be in the vicinity of the Cu-interacting site were determined by manual interpretation of the product ions derived from both MS/MS and MS/MS/MS analysis of peptide fragments (Bridgewater et al., 2006, 2006). Also, specific surface histidine residues of the cuproprotein ceruloplasmin have been shown to coordinate Cu and promote the oxidation of low density lipoprotein (Mukhopadhyay et al., 1997). Our analyses to date have not provided evidence for a link between metal binding sites and MCO in the case of the plant mitochondrial aconitase. However, studies of complex lysates have yet to be performed in any system to demonstrate MCO specificity on a large scale and thus determine if this concept is the exception or the rule in understanding metal-induced damage to proteins.

While it is generally considered that oxidative modification leads to protein dysfunction by affecting the structural integrity of the protein, promoting the formation of protein aggregates, and potentially damaging the active site (Starke-Reed and Oliver, 1989), protein oxidation may not be entirely detrimental to protein function. Hence, among the protein oxidation events in plant mitochondria may be important triggers for the stress response of the organelle. Protein oxidation can promote protein degradation and turnover (Rivett, 1985; Davies and Lin, 1988; Marcillat et al., 1988). It can also provide protective outcomes for the cell, for example, a bacterial transcription factor has been identified that senses and promotes an appropriate cellular response to an oxidative environment through MCO (Lee and Helman, 2006). In addition, methionine residues on protein surfaces has been shown to act as antioxidants to protect the active site of enzymes (Levine et al., 1996) and oxidation of methionine residues can block phosphorylation-induced regulation of proteins (Hardin et al., 2009).

This study attempted to better define the protein interactions with metal ions and the associated modulation of protein functions in plant mitochondria. While the site specificity of MCO could not be confirmed, it was demonstrated that the metal content of mitochondria is dynamic and changes during oxidative stress, that different proteins have varying metal affinity, varying susceptibility to inactivation by H₂O₂,
metal ions, or MCO and that oxidative treatments do not equally and/or detrimentally affect a variety of mitochondrial enzyme activities. Defining the sites of oxidation and mapping them to the functional regions of protein sequences en masse in the future will be essential to uncover the broader oxidative modulation of enzyme activities in plant mitochondria.
Methods

Induction of oxidative stress in Arabidopsis and isolation of mitochondria

Seven-day old dark-grown heterotrophic Arabidopsis thaliana (cv. Landsberg erecta) cell suspension cultures (May and Leaver, 1993) were treated with either 10 mM H₂O₂, 400 μM menadione prepared in methanol, or 25 μM antimycin A prepared in methanol for 8 hours. The concentrations of antimycin A and menadione have previously been optimised (Winger et al., 2005). Equivalent volumes of either water or methanol were added as controls for respective treatments. Mitochondria were isolated from as described previously (Millar et al., 2001).

ICP-MS analysis of metal content

To determine sites of altered metal content, mitochondria were fractionated into soluble protein and integral membrane protein fraction. Intact isolated mitochondria were suspended in milliQ water before lysis by 3 freeze/thaw cycles. Soluble proteins were collected in the supernatant following centrifugation at 20,000 x g. Peripherally attached proteins were depleted from the total membrane fraction using 0.1 M Na₂CO₃ treatment to produce the integral membrane protein fraction (Fujiki et al., 1982). Re-distilled concentrated HNO₃ (kindly provided by Prof. John Watling, Centre for Forensic Science, UWA) was used to breakdown organic material by heating at 160°C for 1 hr. The acid digest was diluted to <5% (v/v) HNO₃, and passed through 0.22 μm filters (Millipore). Metal speciation and quantification was performed by Centre for Forensic Science, UWA using Perkin Elmer Elan 5000 ICP-MS calibrated against elements of interest.

Respiratory assays

Oxygen consumption by cell suspension cultures and isolated mitochondria was measured by a Clark-type oxygen electrode (Hansatech Instruments, UK). Respiratory data were collected and analysed using OxyGraph Plus v1.01 software (Hansatech Instruments, UK). Liquid phase calibration was performed by adding excess sodium dithionite to 1 ml autoclaved water to remove all oxygen at 25°C. Mitochondrial respiration assays were conducted at 25°C by adding 100-120 μg
mitochondrial protein to 1 ml respiration medium (0.3 M sucrose, 5 mM KH$_2$PO$_4$, 10 mM TES, 10 mM NaCl, 2 mM MgSO$_4$, 0.1% (w/v) BSA, pH 7.2). Respiration via Complex II was initiated by adding succinate and ATP. Respiration initiated through the external NADH dehydrogenase pathway was measured using NADH, CaCl$_2$, and rotenone. Respiration initiated through matrix NADH dehydrogenases was measured using malate, glutamate, CoA, TPP, and NAD$^+$. In all respiratory assays, following the addition of substrates to initiate respiration, ADP was added to induce maximum state 3 respiratory rates. Respiration via the alternative and cytochrome oxidase pathway was determined by the addition of the inhibitors KCN and nPG, respectively.

**Enzyme assays**

Assays were performed at 25°C using a temperature controlled spectrophotometer (U-2810 spectrophotometer, Hitachi) and data were collected by UV Solutions software v2.1 (Hitachi). For inhibition assays, mitochondrial samples equivalent to 50 μg of protein were treated with 100 μM of metal ions for 5 min prior to the assay. The metal ions were added at an excess of 1.5 μmol per mg of protein to induce maximal toxicity. Equimolar ascorbate or H$_2$O$_2$ was added to catalyse MCO. The protein samples were then directly added to the reaction medium for enzyme assays. All assays were conducted according to published methods (Lee et al., 2008).

**Statistical analyses**

Unless stated otherwise, all data obtained from experiments were expressed as mean ± standard error about the mean (SEM). Statistical significances were evaluated by the two-tailed unpaired Student’s t-test using Microsoft Office Excel 2003 or Kaleidagraph v3.6 (Synergy Software) where appropriate.

**Immobilized metal affinity chromatography**

HiTrap Chelating HP 1 ml column (Amersham Biosciences) consisting of pre-packed iminodiacetic acid conjugated to agarose beads was used. All chromatography was conducted manually using syringes with the flow rate maintained at approximately 1 ml/min. The column was charged with 1 column volume (CV) of 0.1 M metal ion solution and excess metals were removed with 5 CV of milliQ water. The charged column was equilibrated with 10 CV of binding buffer (20 mM NaH$_2$PO$_4$, 0.5
M NaCl, 0.1% (v/v) Triton X-100) unless stated otherwise. Prior to sample loading, 2 mg of pelleted mitochondrial proteins were suspended in 500 μl of milliQ water and subjected to 3 freeze/thaw cycles. The lysate was passed through 0.22 μm filter (Millipore) and 500 μl of 2-fold concentrated binding buffer was added to the filtered lysate so that the proteins were suspended in 1 ml lysate. The lysate was injected into the column, allowed to incubate at RT for 1-5 min, and then washed with at least 10 CV of binding buffer to remove unbound proteins. Proteins were fractionated with varying concentrations of NH₄Cl or imidazole in the binding buffer. All remaining proteins were removed from the column by stripping the metal ions with an eluent containing 50 mM EDTA and 50 mM NaCl. Due to the high salt content of the eluent fractions, the proteins were concentrated using 5 kDa molecular weight cut-off centrifugal filter units (Millipore) and the excess salt diluted following buffer exchange with milliQ water.

**Metal-binding motif analysis**

Metal-binding motif analysis used was based on analysis of similar work on Arabidopsis root Cu-binding proteins where histidine, methionine and cysteine residues in any combination and up to 12 amino acids apart were considered putative metal-binding motifs (Kung et al., 2006). The algorithm for motif screening was written in MySQL and 117 potential metal-binding motifs were analysed. The gene accession codes of the Arabidopsis proteome, a total of 26738 putative proteins, were extracted from TAIR. The list of accession codes were obtained from SUBA where parameters for mitochondrial proteins were “annotated in SwissProt” or “found in mitochondria by mass spectrometry” or “found in mitochondria by GFP” culminating in 742 proteins (Heazlewood et al., 2005). The standard normal distribution (Z score) statistical analysis was conducted on the frequency of occurrence of the IMAC motifs comparing to the entire Arabidopsis and the Arabidopsis mitochondrial proteomes. Removal of IMAC-interacting proteins from the Arabidopsis and Arabidopsis mitochondrial proteome list was performed in order to conduct statistical analyses on 2 independent samples.

**Metal-catalysed oxidation of proteins**
The Cu ion / ascorbate / O₂ system is the preferred mechanism for metal-catalysed oxidation (MCO) as the major product of this MCO system is the formation of 2-oxohistidine (Uchida and Kawakishi, 1986). Dose response assays determined that 1 nmol metal ion per milligram of protein was optimum to induce maximal metal-catalysed protein oxidation with minimal protein degradation. Equimolar ascorbate was added to catalyse MCO. All MCO treatments were performed at RT with constant agitation for 5 min. The MCO reaction was stopped by the addition of 5 mM EDTA. The metal ions were then removed from the proteins by overnight dialysis at 4°C against 50 mM HEPES-NaOH, pH 7.2.

**In-gel protein digestion and peptide extraction**

Gel plugs from protein bands of interest were excised and de-stained twice for 45 minutes with 50% (v/v) acetonitrile in 25 mM NH₄HCO₃. The plugs were dehydrated at 50°C on a dry block heater for 30 minutes and re-hydrated with 12.5 μg/ml trypsin in 25 mM NH₄HCO₃ and incubated overnight at 37°C. Peptides were extracted by adding 15 μl acetonitrile with vigorous shaking for 15 minutes, removing liquid and secondly, adding 15 μl of 50% acetonitrile and 5% formic acid to the gel plugs followed by another 15 minutes of shaking. The second extraction step was repeated and all the samples were pooled after each extraction step and lyophilised.

**Peptide mass spectrometry and protein identification**

Samples extracted from in-gel trypsin digestions were loaded onto self packed Microsorb (Varian) C18 (5 μm, 100 Å) reverse phase columns (0.5 x 50 mm) using an Agilent Technologies 1100 series capillary liquid chromatography system and eluted into a XCT Ultra IonTrap mass spectrometer with an ESI source equipped with a low flow nebuliser in positive mode and controlled by Chemstation (Rev B.01.03 [204]: Agilent Technologies) and MSD Trap Control v 6.0 (Build 38.15) software (Bruker Daltonik GmbH). Peptides were eluted from the C18 reverse phase column at 10 μL/min using a 9 minute acetonitrile gradient (5 - 60 %) in 0.1 % formic acid at a regulated temperature of 50°C. The method used for initial ion detection utilized a mass range of 200 - 1400 m/z with scan mode set to Standard (8100 m/z per sec) and a Ion Charge Control (ICC) conditions set at 250000 and 3 averages taken per scan. Smart mode parameter settings were employed using a Target of 800 m/z, a
Compound Stability factor of 90%, a Trap Drive Level of 80% and Optimize set to Normal. Ions were selected for MS/MS after reaching an intensity of 80000 cps and two precursor ions were selected from the initial MS scan. MS/MS conditions employed SmartFrag for ion fragmentation, a scan range of 70 - 2200 m/z using an average of 3 scans, the exclusion of singly charged ions option and ICC conditions set to 200000 in Ultra scan mode (26000 m/z per sec). Resulting MS/MS spectra were exported from the DataAnalysis for LC/MSD Trap version 3.3 (Build 149) software package (Bruker Daltonik GmbH) using default parameters for AutoMS(n) and compound Export. For identification of proteins excised from SDS-PAGE gels, spectra were queried against the Arabidopsis protein set (TAIR version 7.0) using the Mascot search engine (Matrix Science) (Perkins et al., 1999) utilising error tolerances of ± 1.2 Da for MS and ± 0.6 Da for MS/MS, ‘Max Missed Cleavages’ set to 1, with variable modifications of Oxidation (M) unless stated otherwise. Instrument set to ESI-TRAP and ‘peptide charge’ set at 2+ and 3+. Results were filtered using ‘standard scoring’, ‘Max. number of hits’ set to 20, ‘Significance threshold’ at p< 0.05 and ‘Ions score cut-off’ at 0. The top 3 protein hits were reported unless stated otherwise.

For the identification of post-translational modifications of aconitase, peptide extracts were analysed on an Agilent 6510 Q-TOF mass spectrometer with an HPLC Chip Cube source. The Chip consisted of a 40 nl enrichment column (Zorbax 300SB-C18 5 u) and a 150 mm separation column (Zorbax 300SB-C18 5 u) driven by Agilent Technologies 1100 series nano/capillary liquid chromatography system. Both systems were controlled by MassHunter Workstation Data Acquisition for Q-TOF (ver B.01.02, Build 65.4, Patches 1,2,3,4, Agilent Technologies). Results were queried against the Arabidopsis protein set (TAIR version 7.0) using the Mascot search engine (Matrix Science, UK) utilising error tolerances of ± 1.2 for MS and ± 0.6 for MS/MS, ‘Max Missed Cleavages’ set to 1, with variable modifications of Oxidation (M) unless stated otherwise. Instrument set to ESI-Q-TOF and ‘peptide charge’ set at 2+ and 3+. Results were filtered using ‘standard scoring’, ‘Max. number of hits’ set to AUTO, ‘Significance threshold’ at p< 0.05 and ‘Ions score cut-off’ at 0. MASCOT Daemon v2.0 was used to automate searches using variable modifications already submitted to the Unimod database. The variable modifications of oxidation of the amino acids C, D,
F, K, M, N, P, R, and Y were used to determine if MCO elicited any detectable changes between control and oxidised protein samples.

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Table I. The Arabidopsis cell suspension culture mitochondrial metallome.

A.

| Element | ng metal / mg protein | pmol metal / mg protein | % transition metallome |
|---------|-----------------------|-------------------------|------------------------|
| Co      | 0.3 ± 0.05            | 5.5 ± 0.5               | 0.1                    |
| Cu      | 50 ± 4                | 750 ± 50                | 14.5                   |
| Fe      | 180 ± 10              | 3200 ± 200              | 62.5                   |
| Mn      | 7.0 ± 0.5             | 125 ± 5                 | 2.5                    |
| Mo      | 2.0 ± 1.5             | 20 ± 15                 | 0.4                    |
| Zn      | 70 ± 30               | 1000 ± 500              | 20                     |

B.

| Element | Total     | Soluble     | Integral membrane |
|---------|-----------|-------------|-------------------|
| Cu      | 100 ± 30  | 75 ± 10     | 240 ± 40          |
| Fe      | 200 ± 40  | 200 ± 40    | 600 ± 80          |
| Mn      | 15 ± 3    | 25 ± 5      | 30 ± 1            |

A.) ICP-MS quantification of transition metal content of isolated Arabidopsis cell culture mitochondria. Data are expressed as both ng and pmol of metal per milligram of protein ± SEM where n=3 biological replicates. The numbers presented in % transition metallome is calculated on a molar basis. B.) Metal content of whole mitochondria (total), soluble fraction (soluble) and the Na₂CO₃-treated insoluble fraction (integral membrane) are expressed as ng metal per milligram of protein ± SEM where n=3 biological replicates.
Table II. Changes in the metallome of mitochondria by oxidative stress.

A.

| Treatment       | Cu     | Fe     | Mn     |
|-----------------|--------|--------|--------|
| Control         | 100 ± 20| 180 ± 30| 15 ± 2 |
| H$_2$O$_2$      | 60 ± 10* | 100 ± 15| 15 ± 1 |
| Methanol        | 130 ± 10| 165 ± 10| 20 ± 2 |
| Antimycin A     | 100 ± 10| 400 ± 75*| 20 ± 3 |
| Menadione       | 120 ± 10| 330 ± 30**| 20 ± 2 |

| Treatment       | Cu     | Fe     | Mn     |
|-----------------|--------|--------|--------|
| Control         | 80 ± 5 | 200 ± 30| 25 ± 3 |
| H$_2$O$_2$      | 50 ± 5* | 85 ± 10*| 15 ± 1*|
| Methanol        | 80 ± 10| 120 ± 5 | 18 ± 1 |
| Antimycin A     | 100 ± 20| 130 ± 15| 17 ± 2 |
| Menadione       | 95 ± 10| 150 ± 25| 17 ± 2 |

| Treatment       | Cu     | Fe     | Mn     |
|-----------------|--------|--------|--------|
| Control         | 230 ± 30| 600 ± 75 | 28 ± 1 |
| H$_2$O$_2$      | 130 ± 30*| 400 ± 120| 25 ± 7 |
| Methanol        | 350 ± 75| 300 ± 25 | 25 ± 2 |
| Antimycin A     | 200 ± 20| 600 ± 100| 25 ± 4 |
| Menadione       | 160 ± 20*| 500 ± 100| 25 ± 4 |

B.

| Fraction        | Cu     | Fe     | Mn     |
|-----------------|--------|--------|--------|
| Total           |        |        |        |
| Control         | 60 ± 20| 200 ± 40| 20 ± 4 |
| H$_2$O$_2$      | 40 ± 5 | 120 ± 2*| 15 ± 1 |

| Soluble         |        |        |        |
| Control         | 35 ± 5 | 160 ± 30| 30 ± 5 |
| H$_2$O$_2$      | 35 ± 10| 60 ± 30*| 25 ± 10|

| Integral membrane |        |        |        |
| Control           | 140 ± 20| 160 ± 70| 10 ± 2 |
| H$_2$O$_2$        | 140 ± 40| 260 ± 80| 10 ± 4 |

A.) The metal content of mitochondria isolated from cell suspension cultures treated with chemical elicitors of oxidative stress for 8 hours were determined by ICP-MS. Data are expressed as ng metal per milligram of protein ± SEM where n≥4. Statistical significance is indicated by **, *, # where p<0.01, <0.05, and <0.1 respectively. B.) Isolated mitochondria subjected to no treatment (control) or 100 μM H$_2$O$_2$ treatment for 5 minutes at RT. The mitochondria were then fractionated into total mitochondria, soluble fraction, flow-through of soluble fraction, and integral membrane fraction for
ICP-MS analysis. Data represents the average metal content expressed as ng metal / mg protein (n=3) ± SEM. Statistical significance is indicated by * where \( p<0.1 \).
**Table III. Metal ions inhibition of respiratory rate of isolated mitochondria.**

A. Total respiration

| Substrate          | [Cu²⁺] (µM) | [Zn²⁺] (µM) |
|--------------------|-------------|-------------|
|                    | 10          | 50          | 100         | 10          | 50          | 100         |
| Glutamate/malate   | 105 ± 20    | 60 ± 15     | 60 ± 15     | 70 ± 10     | 75 ± 15     | 60 ± 15     |
| NADH               | 65 ± 20     | 30 ± 1**    | 20 ± 5**    | 80 ± 10     | 50 ± 2**    | 60 ± 2**    |
| Succinate + ATP    | 110 ± 15    | 55 ± 5**    | 30 ± 10**   | 100 ± 10    | 55 ± 5**    | 65 ± 5**    |

B. Cytochrome oxidase-dependent respiration

| Substrate          | [Cu²⁺] (µM) | [Zn²⁺] (µM) |
|--------------------|-------------|-------------|
|                    | 10          | 50          | 100         | 10          | 50          | 100         |
| Glutamate/malate   | 100 ± 20    | 55 ± 20     | 60 ± 20     | 100 ± 15    | 55 ± 15*    | 30 ± 15*    |
| NADH               | 50 ± 25     | 30 ± 15*    | 35 ± 10*    | 70 ± 20     | 55 ± 10*    | 40 ± 5**    |
| Succinate + ATP    | 140 ± 15    | 70 ± 10*    | 35 ± 15*    | 60 ± 20     | 55 ± 5**    | 65 ± 5**    |

C. Alternative oxidase-dependent respiration

| Substrate          | [Cu²⁺] (µM) | [Zn²⁺] (µM) |
|--------------------|-------------|-------------|
|                    | 10          | 50          | 100         | 10          | 50          | 100         |
| NADH               | 85 ± 10     | 20 ± 5**    | 10 ± 10**   | 60 ± 10     | 30 ± 1**    | 60 ± 15     |
| Succinate + ATP    | 130 ± 30    | 15 ± 5**    | 5 ± 5**     | 80 ± 15     | 40 ± 15*    | 45 ± 10**   |

Isolated mitochondria (120 µg) were treated with various concentrations of Cu²⁺ or Zn²⁺ for 2 minutes prior to assaying the respiratory rates. Respiratory substrates glutamate/malate, NADH and succinate/ATP, were added to drive respiration. A.) State 3 respiration was achieved by the addition of ADP and B.) COX-dependent respiration was measured by adding nPG to ensure the rate observed was COX-dependent. C.) AOX-dependent respiration was measured by adding KCN. Normalised data are expressed as % control ± SEM (n≥3, where * = p<0.05 and ** = p<0.01, comparing each sample to the total oxygen consumption in untreated mitochondria). 10, 50, 100 µM concentrations represent metal ion: protein ratios of 83, 415, and 830 nmol per mg mitochondrial proteins, respectively.
Table IV. Modulation of mitochondrial enzyme activities by metal ions and metal-catalysed oxidation.

| Metal ion interaction# | ACON | FUM | KGDH | PDC | MDH | ICDH |
|------------------------|------|-----|------|-----|-----|------|
| Control                | 116 ± 12 | 83 ± 9 | 55 ± 4 | 80 ± 7 | 3100 ± 400 | 32 ± 4 |
| asc                    | 132 ± 10 | 96 ± 10 | 57 ± 5 | 82 ± 8 | 3900 ± 500 | 25 ± 4 |
| H₂O₂                   | 75 ± 9* | 71 ± 17 | 60 ± 2 | 74 ± 5 | 3900 ± 300 | 34 ± 4 |
| Cu²⁺                   | 22 ± 7** | 82 ± 21 | 11 ± 4** | 25 ± 3** | 3300 ± 400 | 37 ± 3 |
| Cu²⁺ + asc             | 29 ± 10** | 91 ± 14 | 14 ± 4** | 30 ± 10** | 2800 ± 300 | 32 ± 3 |
| Cu²⁺ + H₂O₂            | 28 ± 3** | 97 ± 23 | 8 ± 2** | 17 ± 8** | 3100 ± 500 | 30 ± 6 |
| Fe³⁺                   | 128 ± 6 | 48 ± 11* | 56 ± 6 | 73 ± 7 | 3700 ± 500 | 25 ± 3 |
| Fe³⁺ + asc             | 108 ± 8 | 61 ± 5* | 55 ± 4 | 72 ± 14 | 3000 ± 500 | 27 ± 5 |
| Fe³⁺ + H₂O₂            | 103 ± 7 | 43 ± 8** | 53 ± 3 | 79 ± 5 | 2900 ± 400 | 26 ± 4 |
| Zn²⁺                   | 99 ± 7 | 68 ± 14 | 22 ± 3** | 48 ± 2** | 3900 ± 400 | 36 ± 4 |
| Zn²⁺ + asc             | 100 ± 6 | 70 ± 20 | 21 ± 2** | 42 ± 10* | 3500 ± 500 | 25 ± 5 |
| Zn²⁺ + H₂O₂            | 90 ± 11 | 83 ± 17 | 25 ± 3** | 31 ± 12** | 4100 ± 400 | 37 ± 7 |

Data are expressed as the average nmol product min⁻¹ mg⁻¹ protein ± SEM (n≥4), where * = p<0.05 and ** = p<0.01, comparing each treatment to the appropriate enzyme activity in untreated mitochondria. The measured product was aconitate for ACON, fumarate for FUM, and NADH for KGDH, PDC, MDH, and ICDH. #Metal ion interactions as determined by IMAC experiments. Metal ions were added at 1.5 µmol per mg of mitochondrial protein.
**Figure 1. IMAC affinity purification of proteins from Arabidopsis mitochondria.**

A) selective purification of mitochondria protein subsets using different metals to charge IMAC resin and EDTA elution. B) Different strengths of Cu2+ affinity to IMAC using NH4Cl step gradient elution. C) Different strength of Co2+ and D) Zn2+ using imidazole gradient elution. E) Venn diagram of proteins identified from each metal binding set using mass spectrometry (See Tables S1-S4 for details).

**Figure 2. Metal-binding motif analysis of proteins.** Combinations of histidine (H), cysteine (C) and methionine (M) residues within proximity of 12 amino acid residues were considered putative metal-binding motifs. X represents any amino acid and n denotes number of amino acids apart. The frequency of motifs occurring in A, Cu2+-IMAC isolated subset (n=36 proteins) [blue], B, Co2+-IMAC isolated subset (n=27 proteins) [pink], and C, Zn2+-IMAC isolated subset (n=36 proteins) [yellow] were compared to that of the Arabidopsis proteome (n=26702 proteins) [green], and the Arabidopsis mitochondrial proteome (n=716 proteins) [orange]. The top 6 statistically significant motifs are highlighted in the red boxes.

**Figure 3. Peptides identified for plant mitochondrial aconitase (At2g05710).** (A) number of peptides consistently identified by mass spectrometry common to both mock and H2O2 treated enzymes, or unique to each treatment, n=3-4 experiments, peptides noted appeared in at least 2 experiments. B) Mapping identified peptides onto the 3D structure of the mammalian aconitase. C) BLAST alignment of the region containing the oxidized peptide in plant mitochondrial aconitases and human IRP1 (2b3y). Oxidised (red) and active site (green) residues are indicated on B and C. Substrate recognition site (yellow) and FeS cluster ligation site (orange) residues are shown in B. In C, * Asp 125 is a conserved active site residue and was found to be oxidised.
Supplementary Tables and Figures

Table S1. List of weak Cu$^{2+}$-IMAC binding proteins eluted at 0.1 M to 0.6 M NH$_4$Cl.

Table S2. List of strong Cu$^{2+}$-IMAC binding proteins eluted at 0.8 M to 1 M NH$_4$Cl and by EDTA.

Table S3. List of Co$^{2+}$-IMAC interacting proteins.

Table S4. List of Zn$^{2+}$-IMAC interacting proteins.

Table S5. Frequency of occurrence of metal binding motifs (a-i)

Table S6. Peptides identified from control and oxidized aconitase (a-c)

Figure S1. Annotated ammonium chloride step gradient fractionation of Cu$^{2+}$-IMAC bound proteins.

Figure S2. Annotated imidazole step gradient fractionation of Co$^{2+}$-IMAC bound proteins

Figure S3. Annotated imidazole step gradient fractionation of Zn$^{2+}$-IMAC bound proteins.

Figure S4. Effects of binding buffer pH and ionic strength on protein binding to Fe$^{3+}$-IMAC.

Figure S5. Percentage coverage of IMAC protein sets by identified metal-binding motifs.

Figure S6. Localisation of metal-binding motifs and oxidised residues.

Data S1. Animation showing oxidized peptides and putative copper-binding sites on rotating 3D structure of aconitase.
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