# HMA2. A Transmembrane Zn<sup>2+</sup> Transporting ATPase from

Arabidopsis thaliana

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To my mother and father who opened the door that lead me to a world where the only limitation is my own imagination.

A cloud does not know why it moves in such a direction and at such a speed, it feels an impulsion.....this is the place to go now. But the sky knows the reason and the patterns behind all clouds, and you will know, too, when you lift yourself high enough to see beyond horizons.

Richard Bach

### ABSTRACT

 $P_{1B}$ -type ATPases transport a number of monovalent and divalent heavy metals (Cu<sup>+</sup>, Cu<sup>2+</sup>, Ag<sup>+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup> and Co<sup>+2</sup>) across biological membranes. These ATPases are found in archea, bacteria and eukaryotes and are one of the key elements required for maintaining metal homeostasis. Plants have an unusually high number of  $P_{1B}$ -type ATPases with distinct metal selectivity compared to other eukaryotes that usually have one or two Cu<sup>+</sup>-ATPases. Higher plants are the only eukaryotes where Zn<sup>2+</sup>-ATPases have been identified. Towards understanding the physiological roles of plant Zn<sup>2+</sup>-ATPases, we characterized *Arabidopsis thaliana* HMA2. We expressed HMA2 in yeast and measured the metal dependent ATPase activity in membranes. We showed that HMA2 is a Zn<sup>2+</sup>-ATPase that is also activated by Cd<sup>2+</sup>. Zn<sup>2+</sup> transport determinations showed that this enzyme drives the efflux of metal from the cytoplasm.

Analysis of HMA2 mRNA levels showed that the enzyme is present in all plant organs. We analyzed the effect of removal of HMA2 full-length transcript in whole plants by gene knock out. Although *hma2* mutants did not show a different visible phenotype from the wild type plants, we observed increased levels of  $Zn^{2+}$  or  $Cd^{2+}$ . The observed phenotype of *hma2* mutants and plasma membrane location of HMA2, mainly in vasculature (Hussain et al., 2004), indicates that this ATPase might have a central role in  $Zn^{2+}$  uploading into the phloem.

 $P_{1B}$ -type ATPases have cytoplasmic regulatory metal binding domains (MBDs) in addition to transmembrane metal binding sites (TMBDs). Plant Zn<sup>2+</sup>-ATPases have distinct sequences in both their N- and C-termini that might contribute to novel metal binding sites. These ATPases contain long C-terminal sequences rich in CC dipeptides

and His repeats. Removal of the C-terminus (C-MBD) of HMA2 leads to  $\approx 50\%$ reduction in the enzyme turnover suggesting a regulatory role for this domain. Atomic Absorption Spectroscopy (AAS) analysis showed that  $Zn^{2+}$  binds to C-MBD with a stoichiometry of three (3 Zn/C-MBD). Chemical modification studies and Zn K-edge Xray Absorption Spectroscopy (XAS) of Zn-C-MBD showed that  $Zn^{2+}$  is likely coordinated by His in two sites and the third site slightly differs from the others involving a Cys together with three His. All plant Zn<sup>2+</sup>-ATPases lack the typical CXXC signature sequences observed in Cu<sup>+</sup>-ATPases and some bacterial Zn<sup>2+</sup>-ATPases N-terminus metal binding domains (N-MBDs). Instead, these have conserved CCXXE sequences. Truncation of HMA2 N-MBD results in a 50% decrease in enzyme V<sub>max</sub> suggesting that N-MBD is also a regulatory domain. The results indicate that the N-MBD binds  $Zn^{2+}$ with a stoichiometry of one (1 Zn/N-MBD). Metal binding analysis of individual N-MBD mutants Cys17Ala, Cys18Ala and Glu21Ala/Cys prevented Zn<sup>+2</sup> binding to HMA2 N-MBD suggesting the involvement of all these residues in metal coordination. ATPase activity measurements with HMA2 carrying the mutations Cys17Ala, Cys18Ala and Glu21Ala/Cys showed a reduction in the enzyme activity similar to that observed the truncated protein indicating that the enzyme activity reduction observed in the Nterminus truncated forms of the enzyme is related to the removal of the metal binding capability.

Summaryzing, these studies show the central role of HMA2 in plant  $Zn^{2+}$  homeostasis. They also describe the mechanism and direction of  $Zn^{2+}$  transport. Finally, they establish the presence of novel metal binding domains in the cytoplasmic portion of the enzyme. Metal binding to these domains is required for full enzymatic activity.

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# **ABBREVIATIONS**

AAS: Atomic Absorption Spectroscopy ATP-BD: ATP Binding Domain A-Domain: Actuator Domain CD: Circular Dichroism C-MBD: C-terminus Metal Binding Domain CPM: Coumarine maleimide DEPC: Diethyl pyrocarbonate DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid) EXAFS: X-ray Absorption Fine Structure g.o.f: Goodness of fit IAA: Iodoacetic acid N-MBD: N-terminus Metal Binding Domain N-Domain: Nucleotide Binding Domain P-Domain: Phosphorylation Domain **RT**: Reverse Transcription TCEP: Tris(2-carboxyethyl)phosphine TMBD: Transmembrane Metal Binding Domain XANES: X-ray Absorption Near-Edge Structure XAS: X-ray Absorption Spectroscopy

Nothing happens by chance, my friend....No such thing as luck. A meaning behind every little thing, and such a meaning behind this. Part for you, part for me, may not see it all real clear right now, but we will before long. Richard Bach (from "Nothing by Chance")

### **1. INTRODUCTION**

#### **1.1 Metals in Biological Systems**

Organisms require essential heavy metals including Cu, Zn, Mn, Fe, Co, Ni and Mo to carry out biological functions. In biological systems, these metals are mostly bound to proteins. In these metalloproteins, they have catalytic and structural roles: 1) as constituents of enzyme active sites; 2) stabilizing enzyme tertiary or quaternary structure; 3) forming weak-bonds with substrates contributing to their orientation to support chemical reactions; and 4) stabilizing charged transition states (Fraga, 2005). Cu, Fe and Mn have unpaired electrons that allow their participation in redox reactions in enzyme active sites (Fraga, 2005). For instance, Cu mediates the reduction of one superoxide anion to hydrogen peroxide and oxidation of a second superoxide anion to molecular oxygen in the active site of cytoplasmic superoxide dismutase (Roberts et al., 1991). Zn does not have any unpaired electrons in the  $Zn^{2+}$  state and it has been proposed to prevent the formation of harmful free radicals by competing with the redox active metals such as Fe and Cu in the enzyme active sites (Tapiero and Tew, 2003). For example, it has been observed that Zn<sup>2+</sup> antagonizes Fe mediated xanthine oxidase induced peroxidation resulting in a decrease in the formation of  $O_2^-$  and 'OH (Afanas'ev et al., 1995).  $Zn^{2+}$  is also a cofactor of a number of enzymes including RNA polymerase, carbonic anhydrase and Cu/Zn superoxide dismutase (Fraga, 2005). Other heavy metals including Cd, Pb, Cr, Hg and As have no known physiological activity and are non-essential (He et al., 2005). Elevated levels of both, essential and non-essential heavy metals, results in toxicity symptoms mostly associated with the formation of reactive oxygen species (ROS) that can initiate oxidative damage. Alternatively, they bind to sulphydryl and amino groups in proteins, leading to activity inhibition or structure disruption (Balamurugan and Schaffner, 2006; Gaetke and Chow, 2003; Tapiero and Tew, 2003; Tapiero et al., 2003). Therefore, organisms have tightly controlled homeostatic mechanisms to maintain physiological concentrations of essential heavy metals in different cellular compartments and to minimize the damage from exposure to non-essential ones.

The main mechanisms of heavy metal homeostasis include transport, chelation, and detoxification by efflux or sequestration into organelles, for instance, vacuoles in plants (Clemens, 2001) (Fig. 1.1). Heavy metals are transported into the cells by various transmembrane metal carriers. It has been shown that although cellular  $Zn^{2+}$  or  $Cu^{2+}$  total concentrations are in the millimolar and micromolar range respectively, cytosolic free  $Zn^{+2}$  concentration is in the femtomolar range while free  $Cu^{+}$  is in the zeptomolar range, i.e. less than one free atom per cell (Changela et al., 2003; Outten and O'Halloran, 2001). This indicates that the heavy metals are immediately complexed with molecules or peptides upon entry to the cell. Chelators buffer cytosolic metal concentrations and they involve molecules such as phosphates, phytates, polyphenols and glutathiones, or small peptides such as phytochelatins and some proteins like metallothioneins (Callahan et al., 2006; Fraga, 2005). Some of these chelators are thought to be involved in metal transport into subcellular organelles. For instance, it has been shown that Schizosaccharomyces pombe vacuolar membrane ABC (ATP-binding cassette)-type protein HMT1 transports phytochelatin-Cd<sup>2+</sup> complexes into the vacuole (Ortiz et al., 1995). Chaperones are proteins that bind specific essential heavy metals and deliver them to particular target metalloproteins where they function as part of the enzymatic activity. Similarly, they traffic the metal to specific membrane transporters that efflux the metal to the

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extracellular space and the lumen of subcellular organelles (Clemens, 2001; O'Halloran and Culotta, 2000; Pena et al., 1999). Figure 1.1 summarizes the interplay of metals with these various molecular components.



Figure 1.1 Schematic Representation of Heavy Metal Homeostasis in a Plant

**Cell.** Metal influx to the cytoplasm or efflux to the extracellular space is driven by plasma membrane localized metal transporters. In the cytoplasm, metal ions are bound to chelators that buffer the cytosolic metal concentrations or chaperones that deliver the metal to target proteins. Metal uptake into the subcellular organelles is driven by membrane localized transporters. Inside the subcellular organelles, metals are either delivered to target proteins or stored as metal-chelator complexes.

#### **1.2 Metal Transporters in Plants**

Heavy metal transporting proteins have an important role in the maintenance of metal homeostasis in plants. These are involved in a number of different processes involving metal uptake, delivery of metals to cellular compartments and metal

detoxification through efflux (Colangelo and Guerinot, 2006; Williams and Mills, 2005; Williams et al., 2000). There are several classes of proteins that have been implicated in heavy metal transport. Heavy metal influx proteins drive the influx of the metal to the cytoplasm or remobilize metals from intracellular compartments into the cytoplasm. These involve: 1) the natural resistance-associated macrophage protein (NRAMP) family (Fe<sup>2+</sup>, Cd<sup>2+</sup>, Ni<sup>2+</sup>); 2) the Zrt/IRT-like proteins (ZIP) family (Fe<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>); 3) copper transporter protein (COPT) family (Cu<sup>+</sup>); and 4) the yellow stripe-like (YSL) family of transporters (Fe<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>+</sup>) (Colangelo and Guerinot, 2006; Williams et al., 2000). It should be noted that the putative metal specificities indicated in parentheses are mainly based on functional complementation assays and these have not been characterized by biophysical and biochemical studies. Heavy metal efflux proteins drive the efflux of heavy metals out of the cytoplasm and involve: 1)  $P_{1B}$ -type ATPases (Cu<sup>+</sup>, Ag<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup> and Co<sup>2+</sup>); and 2) the cation diffusion facilitator (CDF) family of transporters (Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>) (Argüello, 2003; Colangelo and Guerinot, 2006; Williams et al., 2000). The following sections will focus on  $P_{1B}$ -type ATPases, the topic of this thesis.

#### **1.3 Structural Features of P<sub>1B</sub>-type ATPases**

 $P_{1B}$ -type ATPases, a subfamily of P-type ATPases, transport a variety of monovalent and divalent heavy metals across membranes using the energy of hydrolysis of the terminal phosphate bond of ATP (Axelsen and Palmgren, 1998, 2001; Møller, 1996). They are thought to appear in early evolution and are key proteins in the maintenance of metal homeostasis in a number of organisms including archea, bacteria,

fungi, and eukarya (Axelsen and Palmgren, 1998, 2001; Colangelo and Guerinot, 2006; Williams and Mills, 2005; Williams et al., 2000).

Analysis of  $P_{1B}$ -type ATPases sequences suggests that most have 8 transmembrane helices (TM) (Fig. 1.2) (Argüello, 2003; Axelsen and Palmgren, 1998, 2001; Bull and Cox 1994; Lutsenko and Kaplan 1995; Solioz and Vulpe 1996). However, a small subgroup of P<sub>1B</sub>-type ATPases appears to have 6 TMs (Argüello, 2003). The presence of 8 TMs has been experimentally confirmed for two bacterial enzymes, Helicobacter pylori CadA and Staphylococcus aureus CadA (Melchers et al., 1996; Tsai et al., 2002). The conserved residues in TMs H6, H7 and H8 form the transmembrane metal binding domain (TMBD) and provide signature sequences that predict the metal selectivity of P<sub>1B</sub>-type ATPases (Argüello, 2003; Mandal et al., 2004). A large cytoplasmic loop responsible for ATP binding and hydrolysis is located between TMs H6 and H7. This loop, referred to as ATP binding domain (ATP-BD), encompasses the nucleotide binding (N) and phosphorylation (P) domains (Fig. 1.2) (Sazinsky et al., 2006b). These two domains are separated by a "hinge" region. The smaller cytoplasmic loop between TM H4 and H5 forms the actuator (A) domain (Sazinsky et al., 2006a). In a  $P_2$ -type sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase, SERCA1, this loop has been shown to interact with the P-domain during the catalytic cycle (Olesen et al., 2004; Toyoshima and Inesi, 2004; Toyoshima and Nomura, 2002; Toyoshima et al., 2004). In addition to the TMBD, most  $P_{1B}$ -type ATPases have regulatory cytoplasmic metal binding domains located in the N-terminus (N-MBDs), C-terminus (C-MBDs), or both. Most N-MBDs are characterized by one to six copies of a highly conserved domain containing the CXXC sequence (Argüello, 2003; Arnesano et al., 2002; Lutsenko and Petris, 2003; Rensing et al., 1999). These Cys residues can bind both monovalent and divalent cations ( $Cu^+$ ,  $Cu^{2+}$ ,  $Zn^{2+}$  and  $Cd^{2+}$ ) (Banci et al., 2002; DiDonato et al., 1997; Gitschier et al., 1998; Harrison et al., 1999; Jensen et al., 1999; Lutsenko et al., 1997). In some cases these sequences are replaced by His repeats or other diverse sequences (Argüello, 2003; Axelsen and Palmgren, 2001).



Figure 1.2 Schematic Representation of the Membrane Topology of  $P_{1B}$ -type ATPases. Transmembrane helices, H1-H8, are indicated. The relative locations and structure of *Archaeoglobus fulgidus* CopA actuator (A) domain and phosphorylation (P) and nucleotide (N) domains (Sazinsky et al., 2006a; Sazinsky et al., 2006b) are shown. To represent one of the repeats present in the N-terminus the human Menkes disease protein (MNK) fifth N-terminal metal binding domain (N-MBD) (Banci et al., 2005) is depicted. The conserved amino acids in H6, H7 and H8 forming the transmembrane metal binding sites (TMBDs) are symbolized by red dots. The C-terminal metal binding domains (C-MBDs) with likely diverse structures are represented by yellow rectangles.

#### **1.4 Catalytic Mechanism of P<sub>1B</sub>-type ATPases**

Active transport of the metal by P<sub>1B</sub>-type ATPases follows the E1-E2 Albers-Post model by alternating the affinities of intracellular metal binding sites from high (E1) to low (E2) (Fig. 1.3) (Post et al., 1972). In the E1 state the ATPase has a high affinity for the metal and the TMBDs are accessible only from the cytoplasmic side. In contrast, an enzyme in the E2 state has low affinity for the metal and in this conformation the metal binding site faces the opposite side of the membrane. According to this model, the enzyme in E1 state is phosphorylated by Mg-ATP (µM) with metal ion binding to the TMBD from the cytoplasmic side (E1.ATP. $nM^{+n}$ ). The phosphorylation occurs with the transfer of the terminal phosphate of ATP to a conserved Asp residue located in the Pdomain followed by the subsequent release of ADP (E1.P.nM<sup>+n</sup>). This phosphorylation causes occlusion of the bound metal ion at the TMBD. The enzyme is unstable in the E1.P state and converts rapidly to the E2.P state. This transition leads to the release of the metal ions into the extracellular/luminal compartment. Finally, dephosphorylation takes place and the enzyme returns to the unphosphorylated and metal free from (E2). The enzyme then returns to the E1 conformation upon ATP (mM) binding to E2.

Biochemical studies with eukaryote, prokaryote and archeal  $P_{1B}$ -type ATPases have provided evidence for individual steps of the catalytic mechanism. ATPase activity, phosphorylation, dephosphorylation and metal transport studies have been carried out with isolated or membrane preparations of Cu<sup>+</sup>- and Zn<sup>2+</sup>-ATPases (Eren and Argüello, 2004; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Mandal et al., 2002; Sharma et al., 2000; Tsivkovskii et al., 2002; Voskoboinik et al., 1998).



**Figure 1.3 Catalytic Mechanism of P**<sub>1B</sub>**-type ATPases.** E1 and E2 represent the different conformations of the enzyme.  $M^{+n}$  represent the different metals that are transported by P<sub>1B</sub>-type ATPases. n indicates the uncertainty on the stoichiometry of the metal transport.  $M^{+n}$  (in) represents the cytoplasmic and  $M^{+n}$  (out) represents the extracellular or luminal localization of the transported metal.

Transport experiments indicate that  $P_{1B}$ -type ATPases drive the metal efflux from the cytoplasm (Eren and Argüello, 2004; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Rensing et al., 1997; Voskoboinik et al., 1998). This is in agreement with a mechanism where the enzyme binds to ATP and the metal in the E1 state (TMBDs are open to the cytoplasmic site). Some earlier reports suggested that some Cu<sup>+</sup>-ATPases might drive metal influx into the cytoplasm (Odermatt et al., 1993; Tottey et al., 2001). However, this would require an alternative mechanism where the binding of another substrate would be required in the E1 state to trigger ATP hydrolysis and enzyme phosphorylation followed by subsequent conformational changes to allow metal influx.

The stoichiometry of transport has not been determined for any of the  $P_{1B}$ -type ATPases. However, a study of *Escherichia coli*  $Zn^{2+}$ -ATPase ZntA has shown that  $Zn^{2+}$  binds to the TMBD with a stoichiometry of 1 metal bound per enzyme (Liu et al., 2006). Although it can be argued whether all the TM metal binding sites were occupied, this is the first study towards determination of stoichiometry of metal transport by  $P_{1B}$ -type ATPases.

# **1.5 Transmembrane Metal Binding Sites and Classification of P**<sub>1B</sub>-Type ATPases

Structural and functional characterization of  $Ca^{2+}$  and  $Na^+/K^+$ -ATPases of P<sub>2</sub>type ATPases indicate that conserved amino acids in their TMs H4, H5 and H6 are responsible for ion binding during transport (Argüello and Kaplan, 1994; Argüello and Lingrel, 1995; Argüello et al., 1996; Argüello et al., 1999; MacLennan et al., 1998; Pedersen et al., 1998; Pedersen et al., 1997; Toyoshima et al., 2000; Vilsen and Andersen, 1998). Sequence alignment and homology studies show that TMs H6, H7 and H8 of P<sub>1B</sub>-type ATPases are structurally similar to P<sub>2</sub>-type ATPases TMs H4, H5 and H6 (Argüello, 2003; Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995). Most P<sub>1B</sub>type ATPases contain a CPX signature sequence in their TM H6 where the Pro is conserved in all P-type ATPases. This sequence has been proposed to participate in metal binding and transport (Argüello, 2003; Axelsen and Palmgren, 1998; Bull and Cox, 1994; Lutsenko and Kaplan, 1995; Rensing et al., 1999; Solioz and Vulpe, 1996). Mutations in the CPC sequence of some Cu<sup>+</sup>-ATPases including *Caenorhabditis elegans* CUA-1, *E*. *coli* CopA, *Archaeoglobus fulgidus* CopA and *Saccharomyces cerevisiae* Ccc2p yielded proteins that were either unable to complement for the Cu<sup>+</sup>-ATPase deficient yeast mutant  $\Delta ccc2$  or had no ATPase activity (Fan and Rosen, 2002; Lowe et al., 2004; Mandal and Argüello, 2003; Yoshimizu et al., 1998). For some of these proteins, it was shown that these still bind to ATP but are unable to hydrolyze it, suggesting that the turnover of the enzyme is prevented by the lack of metal binding to the TMBD (Fan and Rosen, 2002; Lowe et al., 2004; Mandal and Argüello, 2003). Alternative sequences (SPC, CPS, CPT, CPA, CPG, CPD and CPH) have also been observed in some P<sub>1B</sub>-type ATPases. P<sub>1B</sub>-type ATPases contain conserved amino acid residues in TMs H7 and H8 which were proposed to form transmembrane metal binding domains (TMBDs) together with CPX sequences in the TM H6 and determine the metal specificity of these enzymes (Argüello, 2003). The signature sequences in TMs H6, H7 and H8 allow the classification of P<sub>1B</sub>-type ATPases into 5 subgroups with distinct metal selectivity and functional characteristics (Fig. 1.4).

#### Subgroup 1B-1

This group involves the  $Cu^+/Ag^+$ -ATPases. Some of the  $Cu^+$  transporting members of this group have been well characterized. Proteins belonging to this subgroup are found in eukaryotes, prokaryotes and archea. These include Menkes and Wilson disease proteins which are associated with genetic Cu transport disorders in humans (Bull and Cox 1994; Bull et al., 1993; Petrukhin et al., 1994), *Arabidopsis thaliana* RAN1 (Hirayama et al., 1999; Woeste and Kieber, 2000), *E. coli* CopA (Fan and Rosen, 2002; Rensing et al., 2000) and *A. fulgidus* CopA (Mandal and Argüello, 2003; Mandal et al., 2002; Mandal et al., 2004). These  $Cu^+$ -ATPases have been shown to transport nonphysiological substrate  $Ag^+$  and drive the efflux of the metal from the cytoplasm (Fan and Rosen, 2002; Mandal et al., 2002; Rensing et al., 2000).

Proteins in subgroup 1B-1 have a conserved CPC sequence in TM H6 (Fig. 1.4). In addition, these proteins contain the conserved residues, Asn, Tyr in TM H7 and Met, Ser in TM H8. The participation of these residues in metal transport have been shown by site directed mutagenesis studies in AfCopA (Mandal et al., 2004). Mutant AfCopAs were not phosphorylated by ATP in the presence of Cu<sup>+</sup> and therefore were inactive. However, these were phosphorylated by inorganic phosphate (P<sub>i</sub>) indicating that they retained the overall structure and could undergo major conformational transitions. These observations suggested the participation of conserved residues (two Cys of conserved CPC and Asn, Tyr, Met and Ser in H7 and H8) in Cu<sup>+</sup> transport by P<sub>1B</sub>-type ATPases.

#### Subgroup 1B-2

This group involves the  $Zn^{2+}$ -ATPases. Interestingly, these ATPases have been found in archaea, prokaryotes and plants, but not in other eukaryotes (Argüello, 2003). Proteins in this subgroup also have the conserved CPC sequence in TM H6 (Fig. 1.4). However, conserved residues in TM H7 and H8 are clearly distinct from that of subgroup 1B-1 proteins. Subgroup 1B-2 ATPases contain a conserved Lys in TM H6 and Asp and Gly in TM H8. Mutations of Asp714 (Asp714His and Asp714Glu) of *E. coli* ZntA yielded inactive enzymes that were still able to bind Zn<sup>2+</sup> and undergo phosphorylation in the presence of P<sub>i</sub> (Dutta et al., 2006). However, mutant proteins could not be phosphorylated by ATP in the presence of Zn<sup>2+</sup> supporting that conserved residues in TMs H6, H7 and H8 contribute to TMBD.



250 200 150 100 50 0

**Figure 1.4 Phylogenetic Tree of the**  $P_{1B}$ **-type ATPases.** The tree was prepared from a Clustal W alignment of representative sequences of  $P_{1B}$ -type ATPases. The relative abundance of sequences from each subgroup has been maintained. The metal specificity and the structural characteristics are indicated next to the subgroup denomination. Amino acids in TMs are proposed to participate in determining metal selectivity. Black blocks represent His-rich N-MBDs; orange blocks, CXXC N-MBDs; and red, His and Cys rich N- and C-MBDs (This figure is published by Argüello et al., 2007).

#### Subgroup 1B-3

Members of this subgroup are Cu<sup>2+</sup>-ATPases that are found in archaea and bacteria but not in eukaryotes. These have a CPH sequence in TM H6 while amino acids in TM H7 and H8 are similar to those in subgroup 1B-2 (Fig. 1.4). These ATPases also transport Cu<sup>+</sup> and Ag<sup>+</sup> (Mana-Capelli et al., 2003; Odermatt et al., 1993; Solioz and Odermatt, 1995). However, studies with AfCopB indicate that Cu<sup>2+</sup> produces five times more activation of this enzyme compared to that driven by Cu<sup>+</sup> (Mana-Capelli et al., 2003). This is not surprising considering that imidazolium (in conserved CPH; a hard Lewis base) prefers to bind Cu<sup>2+</sup> (an intermediate Lewis base) rather than Cu<sup>+</sup> (Argüello, 2003; Mana-Capelli et al., 2003). A mutation in the CPH sequence (CPH  $\rightarrow$  SPH) in *Enterococcus hirae* CopB resulted in lack of activity suggesting probable contribution of this sequence to TMBD (Bissig et al., 2001).

#### Subgroup 1B-4

Members of this group have only 6 putative TMs (Argüello, 2003). In these, the large cytoplasmic loop is located between TM H4 and H5. TM H4 (corresponding to TM H6 of other  $P_{1B}$ -type ATPases) contains a conserved SPC sequence. The substrate specificity of these enzymes has not been characterized. One member of this subgroup,

*Synechocystis* PCC6803 CoaT, seems to be involved in  $Co^{2+}$  transport since disruption of *coat* gene reduced *Synechocystis*  $Co^{2+}$  tolerance and increased cytoplasmic  $Co^{2+}$  levels (Rutherford et al., 1999). Another subgroup 1B-4 protein, *Arabidopsis thaliana* HMA1, is implicated in Cu<sup>+</sup> transport into the chloroplast (Seigneurin-Berny et al., 2006). Further characterization studies of other members are necessary to delineate the metal specificity of subgroup 1B-4 proteins.

#### Subgroup 1B-5

This subgroup includes only a few proteins that appear to be  $P_{1B}$ -type ATPases based on the presence of typical structural characteristics including the cytoplasmic phosphorylation site (DKTGT), an APC, CPC, or CPS sequence and significant sequence similarity (30-40%) to other  $P_{1B}$ -ATPases. Further studies are required to reveal the metal selectivity of these enzymes and the residues that participate in metal coordination.

#### 1.6 The ATP Binding (ATP-BD) and Actuator (A) Domains

The crystal structures of the isolated N-domain of human Cu<sup>+</sup>-ATPase Wilson's disease protein (WNDP) and A-domain and ATP-BD of *Archaeoglobus fulgidus* Cu<sup>+</sup>-ATPase CopA (AfCopA) have been recently solved (Fig. 1.5) (Dmitriev et al., 2006; Sazinsky et al., 2006a; Sazinsky et al., 2006b). AfCopA A-domain shows a 10  $\beta$ -strand core with 2  $\alpha$ -helices connecting the TMs and its folding shows significant similarity to that of the A-domain of SERCA1, the P<sub>2</sub>-type Ca<sup>2+</sup>-ATPase in spite of their little sequence homology (Fig. 1.5A) (Toyoshima and Inesi, 2004; Toyoshima et al., 2000; Toyoshima et al., 2004). In both, the conserved (S/T)GE(P/S) appears to be located at the tip of a solvent accessible loop on the side of the A-domain. In SERCA1, it has been

shown that this loop interacts with the P-domain during phosphorylation/dephosphorylation driving the rotation of the A-domain with subsequent rearrangement of TMs (Olesen et al., 2004; Toyoshima and Inesi, 2004; Toyoshima and Nomura, 2002; Toyoshima et al., 2004). This arrangement results in metal release. Structural similarity of the  $P_{1B}$ -type ATPases A-domain with that of SERCA1 might point to a similar mechanism for metal release.

The ATP-BD domain structure shows that the P- and N-domains are joined by two short loops called the hinged region (Sazinsky et al., 2006b). The P-domain consists of a 6 stranded parallel  $\beta$ -sheet sandwiched between 3 short  $\alpha$ -helices (Fig. 1.5B). This domain contains the conserved DKTGT sequence and shows similar folding to the Pdomain of SERCA1 (Toyoshima et al., 2000).

The N-domains of both AfCopA and WNDP consists of 6 antiparallel  $\beta$ -sheets flanked by 4  $\alpha$ -helices ((Dmitriev et al., 2006; Sazinsky et al., 2006b). Although the Ndomains of both proteins show a basic similar folding to that of SERCA1, the sequence analysis reveals that the ATP binding site of P<sub>1B</sub>-type ATPases is distinct from that of P<sub>2</sub>type ATPases. Structural analysis of the N-domains of WNDP and KdpB, a P<sub>1A</sub>-type ATPase, in the presence of nucleotides shows that these have unique homologous ATP binding sites (Dmitriev et al., 2006; Haupt et al., 2004; Sazinsky et al., 2006b). The residues that participate in nucleotide binding have been identified in WNDP (His1069, Gly1099, Gly1101, Gly1149, and Asn1150) and the involvement of some of these residues in ATP binding is supported by mutagenesis studies in WNDP (Morgan et al., 2004; Tsivkovskii et al., 2003), *Enterococcus hirae* Cu<sup>+</sup>-ATPase CopB (Bissig et al., 2001) and *Escherichia coli* Zn<sup>2+</sup>-ATPase ZntA (Okkeri and Haltia, 1999; Okkeri et al., 2004).



**Figure 1.5 Structure of the A-, P- and N-domains of** *Archaeoglobus fulgidus* **CopA.** Structures in *A* and *B* are presented with the segments that would be proximal to the TMs in the full-protein on the top of the models. *A*, AfCopA A-domain. Location of conserved SGEP sequence is shown. *B*, AfCopA ATP-BD. P-domain and N-domain are labeled. The location of conserved D424 is indicated. An AMP is modeled in the nucleotide binding site. Surfaces of 22 conserved residues predicted to bind AMP are colored by atom type with oxygen *red*, carbon *grey* (Argüello et al., Biometals, 2007).

#### **1.7 Cytoplasmic Metal Binding Domains**

In addition to TMBD most  $P_{1B}$ -ATPases have 1-6 cytoplasmic metal binding domains (MBD) located either in the N-terminus (N-MBD) or C-terminus or both (Tables 1.1 and 1.2). Most typical ones are the N-MBDs observed in Cu<sup>+</sup>-ATPases and bacterial Zn<sup>2+</sup>-ATPases of subgroups IB-1 and IB-2. These are usually 60-70 amino acid domains characterized by a highly conserved CxxC sequence (Argüello, 2003; Arnesano et al., 2002; Lutsenko and Petris 2003; Rensing et al., 1999). Both conserved Cys have been shown to bind both monovalent and divalent cations including Cu<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Cd<sup>2+</sup>. The high resolution structures of several of the Cu<sup>+</sup>-ATPases N-MBDs show a  $\beta\alpha\beta\beta\alpha\beta$  fold that is similar to the well-described Cu<sup>+</sup>-chaperones like human Atox1, yeast Atx1 and prokaryote CopZ (Banci et al., 2002, 2001; Gitschier et al., 1998). N-MBDs have been shown to receive the metal from these chaperones (Hamza et al., 1999; Huffman and O'Halloran, 2000; Larin et al., 1999; Strausak et al., 2003; Wernimont et al., 2000, 2004). So far no Zn<sup>2+</sup>-chaperone has been identified.

Some  $Zn^{2+}$ -ATPases of subgroup IB-2 have His rich MBDs [(Hx)<sub>n</sub> (n=2-3)] alone or together with the typical N-MBDs (Table 1.1). Similar sequences have been observed in ZIP and Cation Diffusion Facilitator (CDF) families located in loops joining TMs (Eng et al., 1998; Paulsen and Saier 1997). In bacterial  $Zn^{2+}$ -ATPase ZntA a unique CCCDGAC motif in the N-terminus has been shown to coordinate Pb<sup>2+</sup> indicating that different metals might occupy different coordination environments in the same protein (Liu et al., 2005). Eukaryotic (plant)  $Zn^{2+}$ -ATPases contain unique sequences in both Nand C-termini. All plant  $Zn^{2+}$ -ATPases lack the typical N-MBDs. In these the conserved CxxC sequences are replaced with CCxSE (x = S,T,P) sequences (Table 1.2).

Туре	Group <sup>1</sup>	Length <sup>2</sup>	Sequence	Protein
CXXC consensus	1B-1 1B-2	60-80	MVKDTYISSASKTPPMERTVRVTGMT <u>CAMC</u> VKSIETAVGSLEGVEEVRVNL ATETAFIRFDEKRIDFETIKRVIEDLGY GV	A. fulgidus CopA
CCXSE consensus	1B-2	90-100	MASKKMTKSYFDVLGI <u>CCTSE</u> VPLIE NILNSMDGVKEFSVIVPSRTVIVVHDT LILSQFQIVKALNQAQLEANVRVTGE TNFK	A. thaliana HMA2
(HX) <sub>n</sub> (n = 2-6)	1B-2	100-150	MNQPVS <u>HEHKHPHDHAHG</u> DDD <u>HGH</u> <u>AAHGHS</u> CCGAKAAPPLVQLSETASA QAQLSRFRIEAMDCPTEQTLIQDKLSK LAGIEQLEFNLINRVLGVRHTLDGTA DIERAIDSLGMKAEPIAAQDDGSASVP QPAKA	<i>P. putida</i> CadA-2
His rich	1B-3 1B-4	30-100	MNNGIDPENETNKKGAIGKNPEEKIT VEQTNTKNNLQE <u>H</u> GKMENMDQ <u>HH</u> T <u>HGHMERH</u> QQMD <u>HGH</u> MSGMD <u>HSH</u> M D <u>H</u> EDMSGMN <u>HSH</u> MG <u>H</u> ENMSGMD <u>H</u> SM <u>H</u> MGNFKQK	E. hirae CopB

Table 1.1 Cytoplasmic N-terminus Metal Binding Domains of P<sub>1B</sub>-ATPases

1 Refers to subgroup classification showed in Figure 1.4.

2 Number of amino acids.

As a part of this thesis, we have studied functional characteristics of these domains in *A. thaliana* HMA2. In addition,  $Zn^{2+}$ -ATPases have unusually long C-termini that are either rich in His or CysCys repeats or both. We have shown that C-terminus of

HMA2 binds three  $Zn^{2+}$  and modification of His with DEPC inhibits metal coordination completely, indicating a unique coordination of  $Zn^{2+}$  in these ATPases (Eren et al., 2006).

Туре	<b>Group</b> <sup>1</sup>	Length <sup>2</sup>	Sequence	Protein
Cys rich	1B <b>-</b> 2	60-280	LNSMTLLREEWKGGAKEDGA <u>C</u> RAT	O. sativa
			ARSLVMRSQLAADSQAPNAADAGA	HMA3
			AGREQTNG <u>C</u> R <u>CC</u> PKPGMSPEHSVVI	
			DIRADGERQEERPAEAAVVAK <u>CC</u> GG	
			GGGEGIR <u>C</u> GASKKPTATVVVAK <u>CC</u> G	
			GGGGGEGTR <u>C</u> GASKNPATAAVVAK	
			<b><u>CC</u></b> SGGGGEGIG <u>C</u> GASKKPTATAVVA	
			K <u>CC</u> GGGGEGTR <u>C</u> AASKKPATAAVV	
			AK <u>CC</u> GGDGGEGTG <u>C</u> GASKRSPPAEG	
			S <u>C</u> SGGEGGTNGVGR <u>CC</u> TSVKRPT <u>CC</u>	
			DMGAAEVSDSSPETAKD <u>C</u> RNGR <u>CC</u>	
			AKTMNSGEVKG	
Cys/His	1B <b>-</b> 2	260-480	MLLLSDK <u>H</u> KTGNK <u>C</u> YRESSSSSVLIA	A. thaliana
rich			EKLEGDAAGDMEAGLLPKISDK <u>HC</u> K	HMA2
			PG <u>CC</u> GTKTQEKAMKPAKASSD <u>H</u> S <u>H</u> S	
			G <u>CC</u> ETKQKDNVTVVKKS <u>CC</u> AEPVD	
			LG <u>H</u> G <u>H</u> DSG <u>CC</u> GDKSQQP <u>H</u> Q <u>H</u> EVQV	
			QQS <u>CH</u> NKPSGLDSG <u>CC</u> GGKSQQP <u>H</u>	
			Q <u>H</u> ELQQS <u>CH</u> DKPSGLDIGTGPK <u>H</u> EG	
			SSTLVNLEGDAKEELKVLVNGF $\underline{C}$ SSP	
			ADLAITSLKVKSDS <u>HC</u> KSN <u>C</u> SSRER <u>C</u>	
			<u>HH</u> GSN <u>CC</u> RSYAKES <u>C</u> S <u>H</u> D <u>HHH</u> TRA	
			<u>H</u> GVGTLKEIVIE	

Table 1.2 Cytoplasmic C-terminus Metal Binding Domains of P<sub>1B</sub>-ATPases

1 Refers to subgroup classification showed in Figure 1.4.

2 Number of amino acids.

Cu<sup>2+</sup>-ATPases of subgroup IB-3 and only a few members of subgroup IB-4 have distinct His rich MBDs instead of the typical N-MBDs (Argüello, 2003). These domains contain His stretches instead of HX repeats.

#### Regulatory Roles of cytoplasmic MBDs

The absence of cytoplasmic MBDs in some  $P_{1B}$ -ATPases suggests that these are likely regulatory domains (Argüello, 2003). Removal of N-MBD by truncation or inhibition of metal binding capability by mutation results in reduced enzyme activity with small or no changes in metal affinity (Bal et al., 2001; Fan and Rosen 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003; Mitra and Sharma, 2001; Voskoboinik et al., 2001, 1999). Our laboratory has shown that N-MBDs of Archaeglobulus fulgidus Cu<sup>+</sup>-ATPase CopA and Cu<sup>+2</sup>-ATPase CopB control the enzyme turnover rate through the rate limiting conformational change associated with metal release/dephosphorylation (Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Other studies have shown the Cu<sup>+</sup> dependent interaction of Wilson's disease protein N-MBDs with the large ATP binding cytoplasmic loop (Tsivkovskii et al., 2001). Studies of the human Cu<sup>+</sup>-ATPases, Menkes and Wilson Disease proteins showed that at least one intact N-MBD is required for targeting of these ATPases to the plasma membrane and a vesicular compartment, respectively (Forbes et al., 1999; Petris et al., 1996; Schaefer et al., 1999; Strausak et al., 1999). Similar to Cu<sup>+</sup>-ATPases, truncation of Zn<sup>+2</sup>-ATPase ZntA N-MBD results in a decrease in overall rate of the enzyme without altering metal binding affinity (Liu et al, 2006; Mitra and Sharma, 2001). As a part of this thesis, we have shown that truncation of either the N-MBD or C-MBD of A. thaliana  $Zn^{+2}$ -ATPase HMA2 results in  $\approx 50\%$ 

decrease in enzyme activity with no significant change in metal affinity suggesting that both are regulatory domains (Eren et al., 2006).

#### **1.8 Physiological Roles of P<sub>1B</sub>-Type ATPases**

P<sub>1B</sub>-type ATPases were first identified in bacteria like *Staphylococcus aureus* plasmid pI258 (Nucifora et al., 1989), Rhizobium meliloti (Kahn et al., 1989), Escherichia coli (Rensing et al., 2000), Enterococcus hirae (Odermatt et al., 1993) and Synechococcus PCC 6803 (Tottey et al., 2001). Gene knockout studies with most bacterial P<sub>1B</sub>-ATPases resulted in sensitivity of bacteria to high concentrations of metals indicating these ATPases have roles in maintaining metal quotas in the organism (Odermatt et al., 1993; Phung et al., 1994; Rensing et al., 2000; Rensing et al., 1997; Rutherford et al., 1999; Tottey et al., 2001). These studies together with functional complementation assays enabled the initial determination of substrate specificity of P<sub>1B</sub>type ATPases. Cu<sup>+</sup>-ATPases, Zn<sup>2+</sup>-ATPases and a Co<sup>2+</sup>-ATPase have been identified in bacteria. Interestingly, functional and biochemical assays showed that these ATPases can also transport non-physiological substrates. For instance, Cu<sup>+</sup>-ATPases also transport Ag<sup>+</sup> (Fan and Rosen, 2002; Rensing et al., 2000; Solioz and Odermatt, 1995). Similarly, Zn<sup>2+</sup>-ATPases can transport Cd<sup>2+</sup> and Pb<sup>2+</sup> (Rensing et al., 1997; Sharma et al., 2000; Tsai and Linet, 1993).

Similar to their bacterial counterparts, archaeal  $P_{1B}$ -type ATPases present a variety of substrate specificities (Argüello, 2003; Baker-Austin et al., 2005; Mana-Capelli et al., 2003; Mandal et al., 2002). However, these ATPases show diverse properties and considerable structural stability which are linked to the extremophilic character of the hosting organism. For instance, increased Cu<sup>+</sup>-ATPase transcript levels have been

observed in *Ferroplasma acidarmanus*, an organism that tolerates Cu at levels of 20 g/l (Baker-Austin et al., 2005). Another extremophile, *Archaeoglobus fulgidus*, has two  $P_{1B}$ -type ATPases, CopA and CopB, that transport Cu<sup>+</sup> and Cu<sup>+2</sup> respectively, suggesting the need to extrude alternative Cu forms depending on the organism's redox status (Mana-Capelli et al., 2003; Mandal et al., 2002).

In yeast the  $Cu^+$ -ATPase Ccc2p drives  $Cu^+$  export to a late- or post-Golgi compartment in the secretory pathway (Yuan et al., 1997). Exported  $Cu^+$  is eventually incorporated into a multi-copper oxidase Fet3p, which translocates to the plasma membrane and works in conjunction with the iron permease to mediate high affinity Fe uptake (Yuan et al., 1995).

In humans there are two genes (ATP7A and ATP7B) coding for Cu<sup>+</sup>-ATPases: Menkes disease protein (MNKP) and Wilson disease protein (WNDP), that are associated with genetic Cu transport disorders (Bull and Cox, 1994; Bull et al., 1993; Lutsenko et al., 2003; Vulpe et al., 1993). MNKP and WNDP mutant proteins manifest distinct phenotypes due to their differential expression patterns in human tissues. MNKP is expressed in almost all the cells except the hepatic cells. Mutations in MNKP lead to poor Cu uptake from the intestine resulting in severe neurological disorders and connective tissue abnormalities. WNDP is mainly expressed in hepatocytes and mutations in this ATPase result in high Cu levels in the liver, blood and brain causing consequent neurological disorders and cirrhosis. In the cell, both proteins are localized in a trans-Golgi compartment and undergo Cu-dependent trafficking (Hung et al., 1997; Petris et al., 1996). Under conditions of high Cu, MNKP is located to the plasma membrane in various tissues (Petris et al., 1996) while WNDP is targeted to vesicles proximal to the plasma membrane of liver canicular cells where they function in Cu efflux (Forbes et al., 1999; Schaefer et al., 1999).

#### **1.9 Plant P<sub>1B</sub>-Type ATPases**

The focus of our studies has been to understand the functional role and the structural functional relationships of a plant Zn<sup>2+</sup>-ATPase, A. thaliana HMA2. P<sub>1B</sub>-type ATPases have been identified in a number of plants including monocots and dicots (Fig. 1.6) (Argüello, 2003; Colangelo and Guerinot, 2006; Williams et al., 2000). Compared to bacteria, archea or other eukaryotes that usually have one or two Cu<sup>+</sup> transporting P<sub>1B</sub>type ATPases, plants have an unusually high number of P<sub>1B</sub>-type ATPases (eight or nine) with distinct substrate specificities (Argüello, 2003; Williams and Mills, 2005). Most plant Cu<sup>+</sup>-ATPases belong to subgroup 1B-1. Similar to bacterial Cu<sup>+</sup>-ATPases these have a CPC sequence in TM H6 and one to two N-MBDs containing CXXC sequences. Functional complementation studies and sequence homology suggest that plants have several  $Zn^{2+}$ -ATPases that can also confer resistance to other metals including  $Cd^{2+}$  and Pb<sup>2+</sup> when heterologously expressed in yeast or bacteria (Gravot et al., 2004; Hussain et al., 2004; Verret et al., 2004). Our studies showed active transport and direction of  $Zn^{2+}$ efflux by A. thaliana HMA2 (Eren and Argüello, 2004). In all plant Zn<sup>2+</sup>-ATPases the typical CXXC NMBDs have been replaced with conserved CCXSE sequences. Plant Zn<sup>2+</sup>-ATPases also have CC or His rich putative CMBDs. We showed that A. thaliana HMA2 CMBD is a regulatory domain and  $Zn^{2+}$  is mainly coordinated by His and probably some Cys (Eren et al., 2006). Another group of plant P<sub>1B</sub>-type ATPases including A. thaliana HMA1 belongs to subgroup 1B-4. Although it has been suggested
that HMA1 transports  $Cu^+$  (Seigneurin-Berny et al., 2006), the substrate specificity of this group is not clear.



**Figure 1.6 Phylogenetic Tree of the Plant**  $P_{1B}$ **-type ATPases.** The tree was prepared from a Clustal W alignment of representative sequences of plant  $P_{1B}$ -type ATPases from *Arabidopsis thaliana, Oryza sativa, Arabidopsis halleri, Thalaspi caerulescens, Zea mays* and *Sorghum bicolor*. The metal specificities predicted by multiple sequence alignment or determined by functional or biochemical assays are indicated. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. A dotted line on a phenogram indicates a negative branch length.

*A. thaliana* has eight genes encoding for P<sub>1B</sub>-type ATPases (Table 1.3). Multiple sequence alignments indicate that four of them, HMA5, HMA6 (PAA1), HMA7 (RAN1), and HMA8 are closely related to Cu<sup>+</sup>-ATPases. HMA5 appears to be involved in Cu<sup>+</sup> detoxification in roots (Andres-Colas et al., 2006). HMA6 and HMA8 are localized in the chloroplast and are crucial for delivering Cu<sup>+</sup> to key Cu-requiring proteins (Abdel-Ghany et al., 2005; Shikanai et al., 2003). Both HMA6 and HMA8 are involved in Cu<sup>+</sup> delivery to plastocyanin, a Cu-protein that catalyzes electron transfer between the cytochrome  $b_{6}f$ complex photosystem I (PSI) in the thylakoid lumen (Abdel-Ghany et al., 2005). HMA6 also transports Cu<sup>+</sup> across the plastid envelope for incorporation into stromal Cu/Zn superoxide dismutase (SOD) (Abdel-Ghany et al., 2005; Shikanai et al., 2003). HMA7, the first P<sub>1B</sub>-type ATPase to be characterized in plants, is important for the delivery of Cu<sup>+</sup> to receptors of the plant hormone ethylene which is an important regulator of plant growth (Hirayama et al., 1999). An HMA7 mutant (*ran1-3*, responsive to antagonist1-3) was shown to exhibit ethylene phenotypes in response to treatment with transcyclooctene (Hirayama et al., 1999). HMA1 has also been reported to be a Cu<sup>+</sup>-ATPase although its transmembrane metal binding site (TMBD) appears to be different from that of Cu<sup>+</sup>-transporting ATPases (Seigneurin-Berny et al., 2006).

HMA2, HMA3 and HMA4 are  $Zn^{2+}$ -ATPases and have no apparent counterparts in non-plant eukaryotes. As part of this thesis, ATPase activity measurements in yeast membranes expressing HMA2 showed that HMA2 transports  $Zn^{2+}$  and it is also activated by the non-physiological  $Cd^{2+}$  and to a lesser extent by other divalent cations (Eren and Argüello, 2004). Functional complementation assays in bacteria and yeast showed that HMA4 can transport  $Zn^{2+}$ ,  $Cd^{2+}$  and  $Pb^{2+}$  (Mills et al., 2003; Verret et al., 2005). HMA3 confers Cd<sup>+2</sup> and Pb<sup>+2</sup> tolerance to  $\Delta ycf1$  yeast cells and a green fluorescent proteintagged HMA3 appeared to be located located to the yeast vacuole (Gravot et al., 2004). In plant cells both HMA2 and HMA4 are located in the plasma membrane and expressed primarily in the vasculature of shoots and roots (Hussain et al., 2004). The *hma4* mutants present decreased Zn and Cd levels in the leaves while plants overexpressing HMA4 have increased root-to-shoot Zn/Cd translocation (Hussain et al., 2004; Verret et al., 2004). On the other hand, we have shown that *hma2* knockout plants have elevated levels of Zn<sup>+2</sup> (Eren and Argüello, 2004). Considering their distribution, these observations suggest that HMA2 and HMA4 participate in Zn<sup>+2</sup> loading into the phloem and xylem, respectively.

	Tissue Expression	Cellular Localization	Metal Specificity
HMA1	Root, shoot	Chloroplast envelope	$Cu^+$
HMA2	Vasculature of root and shoot	Plasma membrane	$Zn^{2+}, Cd^{2+}$
HMA3	Roots and leaves	Vacuole (?)	$\mathrm{Cd}^{2+}, \mathrm{Pb}^{2+}$
HMA4	Vasculature of root and shoot	Plasma membrane	Zn <sup>2+</sup> , Cd <sup>2+</sup> , Pb <sup>2+</sup>
HMA5	Root, flower	?	$Cu^+$
HMA6	Root, shoot	Chloroplast envelope	$Cu^+$
HMA7	?	Post golgi compartment	Cu <sup>+</sup>
HMA8	Shoot	Thylakoid membrane	$Cu^+$

Table 1.3 Distribution and Metal Specificity of *A. thaliana* P<sub>1B</sub>-ATPases

A scientist in his laboratory is not only a technician: he is a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

# Arabidopsis HMA2, a Divalent Heavy Metal-Transporting P<sub>IB</sub>-Type ATPase, Is Involved in Cytoplasmic Zn<sup>2+</sup> Homeostasis

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## **2.1 ABSTRACT**

 $P_{IB}$ -type ATPases transport heavy metal ions (Cu<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup>, etc.) across biological membranes. Several members of this subfamily are present in plants. Higher plants are the only eukaryotes where putative  $Zn^{2+}$ -ATPases have been identified. We have cloned HMA2, a P<sub>IB</sub>-ATPase present in Arabidopsis (Arabidopsis thaliana), and functionally characterized this enzyme after heterologous expression in yeast (Saccharomyces cerevisiae). HMA2 is a  $Zn^{2+}$ -dependent ATPase that is also activated by  $Cd^{2+}$  and, to a lesser extent, by other divalent heavy metals (Pb<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, and Co<sup>2+</sup>). The enzyme forms an acid-stable phosphorylated intermediate and is inhibited by vanadate. HMA2 interacts with  $Zn^{2+}$  and  $Cd^{2+}$  with high affinity ( $Zn^{2+} K_{1/2} = 0.11 \pm 0.03$  $\mu$ M and Cd<sup>2+</sup>  $K_{1/2} = 0.031 \pm 0.007 \mu$ M). However, its activity is dependent on millimolar concentrations of Cys in the assay media.  $Zn^{2+}$  transport determinations indicate that the enzyme drives the outward transport of metals from the cell cytoplasm. Analysis of *HMA2* mRNA suggests that the enzyme is present in all plant organs and transcript levels do not change in plants exposed to various metals. Removal of HMA2 full-length transcript results in  $Zn^{2+}$  accumulation in plant tissues. *hma2* mutant plants also accumulate  $Cd^{2+}$  when exposed to this metal. These results suggest that HMA2 is responsible for  $Zn^{2+}$  efflux from the cells and therefore is required for maintaining low cvtoplasmic  $Zn^{2+}$  levels and normal  $Zn^{2+}$  homeostasis.

## **2.2 INTRODUCTION**

 $Zn^{2+}$  plays a critical role in plants as an essential component of key enzymes (Cu-Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase, etc.) and DNAbinding proteins (Marschner, 1995; Guerinot and Eide, 1999). Zn<sup>2+</sup> deficiency leads to a reduction of internodal growth with a consequent rosette-like development and also produces an impaired response to oxidative stress, likely due to a reduction in superoxide dismutase levels (Hacisalihoglu et al., 2003). Thus, Zn<sup>2+</sup> deficiency is a significant agricultural problem, particularly in cereals, limiting crop production and quality (Guerinot and Eide, 1999; Hacisalihoglu et al., 2003). Zn<sup>2+</sup> toxicity induces chlorosis in young leaves, probably via competition with Fe<sup>2+</sup> and Mg<sup>2+</sup> (Woolhouse, 1983; Marschner, 1995).

Zn<sup>2+</sup>, as other metal micronutrients, is essential for normal physiology; however, plants must also protect themselves from hazards associated with chemical modifications that these and nonessential metals (Cd<sup>2+</sup>, Pb<sup>2+</sup>, etc.) can drive (Woolhouse, 1983; Williams et al., 2000; Clemens, 2001; Fraústro da Silva, 2001; Hall, 2002). Consequently, plants and other organisms have developed molecular chaperones, chelators, and specific transmembrane transporters to (1) absorb and distribute metal micronutrients throughout the entire organism and (2) prevent high cytoplasmic concentrations of free heavy metals ions (Fox and Guerinot, 1998; Rauser, 1999; Guerinot, 2000; Williams et al., 2000; Clemens, 2001; Cobbett and Goldsbrough, 2002; Hall, 2002). These processes require the metal to be transported through permeability barriers and compartments delimited by lipid membranes. Several types of heavy metal transmembrane transporters have been identified in plants (Rea, 1999; Guerinot, 2000; Maser et al., 2001; Baxter et al., 2003). Since metal ions must be transported against electrochemical gradients at some point during plant distribution, metal pumps involved in contragradient transport should play key roles in metal homeostasis. The presence of plant genes encoding proteins that specifically perform this function (mainly  $P_{IB}$ -ATPases) is known and their potential importance has been repeatedly noted (Williams et al., 2000; Clemens, 2001; Hall, 2002).

P<sub>IB</sub>-ATPases, a subfamily of P-type ATPases, transport heavy metals (Cu<sup>+</sup>, Ag<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>, and Co<sup>2+</sup>) across biological membranes (Lutsenko and Kaplan, 1995; Axelsen and Palmgren 1998; Argüello, 2003). Initial reports named these proteins CPx-ATPases (Solioz and Vulpe, 1996). They confer metal tolerance to microorganisms (Solioz and Vulpe, 1996; Rensing et al., 1999) and are essential for the absorption, distribution, and bioaccumulation of metal micronutrients by higher organisms (Bull and Cox, 1994; Solioz and Vulpe, 1996). Most P<sub>IB</sub>-ATPases appear to have eight transmembrane segments (Melchers et al., 1996; Tsai et al., 2002; Argüello, 2003). Like all other P-type ATPases, they have a large ATP-binding cytoplasmic loop between their sixth (H6) and seventh (H7) transmembrane segments. Within this loop, the Asp in the signature sequence DKTGT is phosphorylated during the catalytic cycle (Lutsenko and Kaplan, 1995; Axelsen and Palmgren, 1998). Two other structural characteristics are usually considered to differentiate P<sub>IB</sub>-ATPases: the signature sequence (CPC, CPH, CPS, SPC, and TPC) in H6, and the frequently present metal-binding domains in the cytoplasmic N-terminal region (Bull and Cox, 1994; Solioz and Vulpe, 1996; Rensing et al., 1999; Argüello, 2003; Lutsenko and Petris, 2003). P<sub>IB</sub>-ATPases appear to have a catalytic mechanism similar to that of well-characterized enzymes in the subgroup P<sub>II</sub> of

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the P-type ATPase family (plant H-ATPase, Na,K-ATPase, sarcoplasmic reticulum Ca-ATPase, etc.). Our group and others have established the formation of the phosphorylated intermediate and basic transport properties for some bacterial, archaeal, and mammalian  $P_{IB}$ -ATPases (Tsai et al., 1992; Tsai and Linet, 1993; Solioz and Odermatt, 1995; Voskoboinik et al., 1998; Rensing et al., 1998a, 1998b; La Fontaine et al., 1999; Okkeri and Haltia, 1999; Voskoboinik et al., 1999; Sharma et al., 2000; Fan and Rosen, 2002; Mandal et al., 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Both in vivo and in vitro metal transport studies have shown that  $P_{IB}$ -ATPases drive the export of ions from the cell cytoplasm (Tsai et al., 1992; Tsai and Linet, 1993; Voskoboinik et al., 1998; Rensing et al., 1992; Tsai and Rosen, 2002; Mandal et al., 2002; Mana-Capelli et al., 2003).

The  $P_{IB}$ -ATPase subfamily includes proteins with different metal specificities (Cu<sup>+</sup>-ATPases, Cu<sup>2+</sup>-ATPases, Zn<sup>2+</sup>-ATPases, Co<sup>2+</sup>-ATPases, etc.) and a particular protein often can transport various metals (Rensing et al., 1999; Argüello, 2003). Functional assays have shown that Ag<sup>+</sup> can also activate Cu<sup>+</sup>-ATPases (Solioz and Odermatt, 1995; Fan and Rosen, 2002; Mandal et al., 2002), Zn<sup>2+</sup>-ATPases can use Cd<sup>2+</sup> and Pb<sup>2+</sup> as substrates (Tsai et al., 1992; Tsai and Linet, 1993; Okkeri and Haltia, 1999; Sharma et al., 2000), and Cu<sup>2+</sup>-ATPases can also be partially activated by Ag<sup>+</sup> and Cu<sup>+</sup> (Mana-Capelli et al., 2003). Although it has been proposed that the Cys-containing sequences in H6 participate in metal binding and transport (Bull and Cox, 1994; Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996; Axelsen and Palmgren, 1998; Rensing et al., 1999; Mandal and Argüello, 2003), the relationship between ion specificity and the various conserved sequences in H6 (CPC, CPH, SPC, TPC, or CPS) is not fully

understood. However, true signature sequences in H6, H7, and H8 have been identified and they appear to be associated with five subtypes of  $P_{IB}$ -ATPases, each with singular metal selectivity characteristics (Argüello, 2003). These conserved amino acids are likely located close to each other, participating in metal coordination and consequently determining the enzyme specificity.

Arabidopsis (Arabidopsis thaliana) has eight genes encoding P<sub>IB</sub>-ATPases (heavy metal ATPases [HMA]). (We have chosen to use the nomenclature recently proposed by Baxter et al. (Baxter et al., 2003) and adopted in the PlantsT database [http://plantst.sdsc.edu/]. Consequently, the proteins previously named PAA1, RAN1, and HMA6 are referred here as HMA6, HMA7, and HMA8, respectively.) A search through other plant genomes (see http://www.tigr.org/ or http://plantst.sdsc.edu) reveals the presence of many putative orthologs (Axelsen and Palmgren, 2001; Baxter et al., 2003). Since other eukaryotes appear to have only two Cu<sup>+</sup>-ATPase isoforms, the presence of multiple, distinct P<sub>IB</sub>-ATPase isoforms seems unique to plants (Argüello, 2003). The analysis of conserved residues in their transmembrane segments suggest that three are Zn<sup>2+</sup>-ATPases (HMA2, 3, and 4) and four are Cu<sup>+</sup>-ATPases (HMA5, 6, 7, and 8; (Argüello, 2003). The remaining protein, HMA1, has different conserved amino acids in the transmembrane region. The only HMA1 homolog that has been studied is CoaT from Synechocystis PCC6803, which appears to confer  $Co^{2+}$  tolerance to this organism (Rutherford et al., 1999). Additionally, the primary sequences of plant  $P_{IB}$ -ATPases suggest the presence of various putative metal-binding domains in their cytoplasmic loops. These are likely to play important regulatory roles, such as those observed in similar domains of other P<sub>IB</sub>-ATPases (Voskoboinik et al., 2001; Mana-Capelli et al.,

2003; Mandal and Argüello, 2003). Several of the plant P<sub>IB</sub>-ATPases, HMA2, HMA3, and HMA4 (putative Zn<sup>2+</sup>-ATPases), and HMA6 and HMA7 (putative Cu<sup>+</sup>-ATPases originally named PAA1 and RAN1), have been the subject of characterization studies (Hirayama et al., 1999; Woeste and Kieber, 2000; Mills et al., 2003; Shikanai et al., 2003; Gravot et al., 2004; Hussain et al., 2004). ran1-1 and ran1-2, two HMA7 mutants, are partially functional and can replace Ccc2 (a Cu<sup>+</sup>-ATPase) in  $\triangle ccc2$  yeast (Saccaromyces cerevisiae; (Hirayama et al., 1999). ran1-1 plants present ethylene phenotypes in response to trans-cyclooctene (ethylene antagonist) (Hirayama et al., 1999). As Cu is part of the ethylene receptor, a decrease in the number of functional receptors was proposed. A third mutant, ran1-3, produces a truncated mRNA and shows constitutive activation of ethylene response pathways and a rosette lethal phenotype. Transgenic 35S::RANI plants show constitutive expression of ethylene response due to cosuppression of RAN1 (Woeste and Kieber, 2000). The requirement to produce functional ethylene receptors suggests a post-Golgi location for HMA7. Arabidopsis mutants that are defective in the HMA6 (PAA1) gene show a high-chlorophyll fluorescence phenotype due to a decrease in holoplastocyanin and a consequent reduction in photosynthetic electron transport (Shikanai et al., 2003). Addition of  $Cu^{2+}$  suppresses this phenotype. These studies suggest that HMA6 is responsible for Cu<sup>+</sup> transport into chloroplasts.

Putative  $Zn^{2+}$ -ATPases present in Arabidopsis are particularly interesting because, as mentioned above, plants are the only eukaryotes where these proteins are present. Initial studies of HMA4 have shown that this enzyme is able to confer cell resistance to high Cd<sup>2+</sup> or Zn<sup>2+</sup> when expressed in yeast or *Escherichia coli*, respectively (Mills et al., 2003). In Arabidopsis, high levels of *HMA4* transcripts are observed mainly in roots and

are increased upon plant exposure to high  $Zn^{2+}$  levels (Mills et al., 2003). Arabidopsis HMA3 confers  $Cd^{2+}$  and  $Pb^{2+}$  tolerance to  $\Delta vcfl$  yeast cells and a green fluorescent protein-tagged HMA3 appears located at the yeast vacuole (Gravot et al., 2004). HMA3 mRNA is detected mainly in roots and its level is not affected in response to exposure to  $Cd^{2+}$  or high  $Zn^{2+}$ . A recent report has shown that, while no morphological alterations were observed in hma2 or hma4 Arabidopsis mutants, an hma2 hma4 double mutant shows visible morphological alterations and male sterility (Hussain et al., 2004). These phenotypes can be compensated by increasing  $Zn^{2+}$  levels in the growth medium. Interestingly, decreased levels of Zn were detected in shoots of the hma2 hma4 mutant and *hma4* single mutant, while increased levels of this metal were detected in roots of the double mutant. In addition, Hussain et al. showed that HMA2 and HMA4 promoters drive the expression of a reporter gene predominantly in vascular tissues and that HMA2 is localized at the plasma membrane in Arabidopsis cells (Hussain et al., 2004). In summary, previous reports have shown the likely role of these proteins in  $Zn^{2+}$ homeostasis and  $Cd^{2+}/Pb^{2+}$  tolerance; however, the molecular properties that determine their physiological functions have not been established.

Toward understanding the physiological roles of  $Zn^{2+}$ -ATPases in plants, we initiated enzymatic and metal transport studies of Arabidopsis HMA2. This article describes the molecular characteristics of this metal-transporting ATPase and its consequent role in metal homeostasis. Ion selectivity, activity rate, direction of transport, catalytic phosphorylation, and interaction with Cys (a P<sub>IB</sub>-type ATPase activator) and vanadate (a classical inhibitor of P-type ATPases) were measured. The presence of *HMA2* mRNA in different plant organs and its variation upon plant exposure to various metals

was determined together with the effect of *HMA2* knockout on  $Zn^{2+}$  homeostasis. The results obtained indicate that HMA2 drives the efflux of  $Zn^{2+}$  from plant cells. Thus, HMA2 appears responsible for maintaining low cellular  $Zn^{2+}$  levels.

## **2.3 RESULTS**

The HMA2 cDNA was amplified by reverse transcription (RT)-PCR. The obtained sequence was identical to that expected from genome sequencing, confirming the intron/exon predictions. The 951-amino acid-long encoded protein shows characteristic features of P<sub>IB</sub>-ATPases (Fig. 2.1). Signature sequences corresponding to Zn<sup>2+</sup>-ATPases are found in H6, H7, and H8, and analysis with topology prediction software suggests that it has eight transmembrane segments (Argüello, 2003). HMA2 contains a relatively short N-terminal end with significant homology to the heavy metal-associated domain (PF00403; (Bateman et al., 2004)). However, it is unlikely that this would have a regulatory role as the typical N-terminal metal-binding domains observed in Cu<sup>+</sup>-ATPases, since a Cys critical for metal binding is not present in HMA2 (CysXXCys -Cys-17CysThrSer-20; (Voskoboinik et al., 1999, 2001; Lutsenko and Petris, 2003). HMA2 has a long C-terminal end (258 amino acids) characterized by the presence of several short sequences that might be involved in heavy metal binding: six CysCys pairs, five HisXHis repeats, and two repeats of the sequence GXDSGCCGXKSOOPHOHEXO (starting at Glv-794 and Glv-824). Supporting the participation of these putative metalbinding sequences in common regulatory mechanisms, several of them can be found in the C-terminal ends of both HMA2 and HMA4 (S<sup>797</sup>GCCG; S<sup>827</sup>GCCG; S<sup>760</sup>SDHSHSGCC: C<sup>930</sup>CRSYAKESCSHDHHHTRAH: positions correspond to the HMA2 sequence).



**Figure 2.1 Structural Features of HMA2.** Transmembrane segments are represented by white rectangles. Numbers in bold indicate the position of transmembrane segments within the HMA2 sequence. Asp-391 is phosphorylated during the catalytic cycle and conserved in all P-type ATPases. Cys-347ProCys, Lys-658, Asp-679, and Gly-681 are conserved in all  $Zn^{2+}$ -ATPases and are likely determinants of the enzyme specificity. Striped and black blocks represent putative metal-binding domains.

#### 2.3.1 Functional Characterization of HMA2

A central point for understanding the role of HMA2 is to elucidate its metal specificity and enzymatic properties. We chose to functionally characterize this enzyme after expressing it in yeast under the control of the *GAL* promoter. Figure 2.2 shows the expression of HMA2 in a membrane vesicle preparation from transformed yeast. HMA2 expression was routinely detected by immunostaining blots with anti-His<sub>6</sub> antibodies. Control experiments showed no differences in metal-dependent ATPase activity or metal

affinity among membrane preparations of the His<sub>6</sub>-tagged HMA2 and those of protein lacking the tag (data not shown). It should be noted that, in the following ATPase and phosphorylation experiments, saponin was included in the assay media to permeabilize sealed vesicles present in the membrane preparations.



**Figure 2.2 Expression of Arabidopsis HMA2 in Yeast.** Membrane preparations from untransformed yeast (lanes 1 and 3) and Gal-induced yeast transformed with HMA2-pYES2/CT (lanes 2 and 4). Lanes 1 and 2, Coomassie Brilliant Blue-stained gel; lanes 3 and 4, blot immunostained with anti-His<sub>6</sub> rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-rabbit IgG-horseradish peroxidase-linked monoclonal antibody (Santa Cruz Biotechnology).

ATPase activity determinations indicate that HMA2 is a  $Zn^{2+}$ -ATPase as expected from its signature sequence in the transmembrane region (Fig. 2.3). HMA2 is also activated by Cd<sup>2+</sup> (97%) and other divalent metals (Ni<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, and Pb<sup>2+</sup>), although to a lesser extent (34%–52%). Even Cu<sup>+</sup> elicits a measurable activity. Confirming that the measured activity was associated with HMA2, no measurable heavy metal-dependent ATPase activity was detected in membranes obtained from yeast transformed with empty pYES2/CT vector. The activity observed in the presence of Ni<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, and Pb<sup>2+</sup> is not surprising since another biochemically characterized  $Zn^{2+}$ -ATPase, *E. coli* ZntA, is also partially activated by these metals, although to different extents (Okkeri and Haltia, 1999; Sharma et al., 2000).



Figure 2.3 Activation of HMA2 ATPase by Metals. HMA2 ATPase activity was determined as indicated in "Materials and Methods." Final concentration of each tested metal ion was 1  $\mu$ M, which is a saturating concentration for all of them. A total of 2.5 mM DTT were included in Cu<sup>+</sup>-containing assay mixture. Bars indicate activity in the presence of each metal as percentage of maximum activity. One-hundred percent = 1.8 to 2.5  $\mu$ mol mg<sup>-1</sup> h<sup>-1</sup>. Values are the mean ± SE (*n* = 4).

The kinetics parameters describing the metal interaction with HMA2 were evaluated by measuring the dependence of ATPase activity on metal concentration (Fig. 2.4). These determinations show that HMA2 is activated by  $Zn^{2+}$  and  $Cd^{2+}$  with surprisingly high affinity ( $Zn^{2+} K_{1/2} = 0.11 \pm 0.03 \ \mu$ M and  $Cd^{2+} K_{1/2} = 0.031 \pm 0.007 \ \mu$ M).



Figure 2.4 Zn<sup>2+</sup> and Cd<sup>2+</sup> Dependence of HMA2 ATPase Activity. The ATPase activity was measured in the presence of different concentrations of Zn<sup>2+</sup> ( $\bigcirc$ ) or Cd<sup>2+</sup> ( $\triangle$ ) ions. Data were fitted using the following parameters: Zn<sup>2+</sup>  $K_{1/2} = 0.11 \ \mu$ M,  $V_{max} = 100\%$ ; Cd<sup>2+</sup>  $K_{1/2} = 0.031 \ \mu$ M,  $V_{max} = 100\%$ . One-hundred percent = 2 to 2.5  $\mu$ mol mg<sup>-1</sup> h<sup>-1</sup>. Values are the mean  $\pm$  SE (n = 4).

In the analysis of the specificity and function of metal transporters, the apparent absence of free heavy metals in living systems should be considered. Both cytoplasmic [Cu] and [Zn] appear to be in the picomolar range under physiological conditions (Rae et al., 1999; Outten and O'Halloran, 2001). In this direction, the dependence of  $P_{IB}$ -ATPase activity on the presence of millimolar Cys in the assay media has been proposed to be an indication of the interaction of the metal complex with these enzymes (Sharma et al., 2000; Mandal et al., 2002). Figure 2.5 shows that HMA2 activity was also dependent on the presence of millimolar Cys in the media, suggesting that plant  $P_{IB}$ -ATPases also appear to require the delivery of complexed metals for activity.



**Figure 2.5 Cysteine Dependence of HMA2 ATPase Activity.** The  $Zn^{2+}$  ( $\bigcirc$ ) and  $Cd^{2+}$  ( $\triangle$ ) dependent ATPase activities were determined as indicated in "Materials and Methods" in the presence of various Cys concentrations. No curve fitting was attempted. One-hundred percent = 2 to 2.5  $\mu$ mol mg<sup>-1</sup> h<sup>-1</sup>. Values are the mean ± SE (*n* = 4).

The unifying functional characteristic of all P-type ATPases is the formation of a phosphorylated intermediate in the presence of ATP-Mg and the outwardly transported substrate (i.e. the ion that is transported out of the cytoplasm into an organelle or the extracellular compartment (Pedersen and Carafoli, 1987). Phosphorylation of HMA2 was performed at 0°C in the presence of micromolar amounts of ATP and 20% dimethyl sulfoxide, conditions that minimize enzyme turnover. Preliminary experiments in which the samples were resolved in acid gels and their radioactivity visualized using a phosphoimager indicated that, under the experimental conditions, a single band corresponding to HMA2 was phosphorylated in the presence of  $Zn^{2+}$  (Fig. 2.6A). Protein phosphorylation was not observed in membranes from yeast transformed with the empty pYES2/CT vector. Similar results were obtained with all tested metals. For simplicity, in subsequent experiments HMA2 phosphorylation was directly quantified counting radioactive emission (Fig. 2.6B). Levels of phosphoenzyme in the presence of various metals roughly follow the activation pattern observed in the ATPase determinations (Fig. 2.3). The small variations are not surprising since these determinations are not equally related to the metal interaction with the enzyme (Mandal et al., 2002; Hou and Mitra, 2003). Figure 2.6B also shows the inhibition of phosphorylation by vanadate, a wellknown feature of P-type ATPases; however, in the case of HMA2, vanadate inhibits its ATPase activity with a slightly high  $IC_{50} = 0.15 \pm 0.05$  mM (not shown). The quantitative determination of phosphoenzyme levels allows the calculation of HMA2 turnover number. Assuming that under the maximum phosphorylation conditions (in the presence of Cd<sup>2+</sup>) most of the enzyme is arrested in the E1P-E2P conformations, and taking into account the activity of the particular HMA2 preparations used in the phosphorylation assays (2  $\mu$ mol mg<sup>-1</sup> h<sup>-1</sup>), a turnover of 143 min<sup>-1</sup> was calculated. This value is similar to those observed in other P<sub>IB</sub>-ATPases (Mandal et al., 2002).



**Figure 2.6 Metal-dependent Phosphorylation of HMA2 by ATP.** A, Membrane preparations from yeast-transformed empty vector (EV) and HMA2-expressing yeast (HMA2) were phosphorylated in the absence (–) or presence (+) of 2.5  $\mu$ m Zn<sup>2+</sup>, resolved by SDS-PAGE in an 8% acidic gel, and visualized in a phosphoimager. HMA2 protein is indicated. B, The metal-activated enzyme phosphorylation by ATP was measured as described in "Materials and Methods." One-hundred percent = 0.232 nmol mg<sup>-1</sup>. Values are the mean ± SE (*n* = 4).

Although the phosphorylation determinations suggest that HMA2 transports metals out of the cytoplasm, it is pertinent to directly demonstrate the direction of metal transport by this enzyme. To this end, the vesicular nature of the yeast membrane preparation can be exploited. Although this preparation might contain broken vesicles, only sealed inside-out vesicles would be able to perform ATP-dependent metal accumulation. Similar yeast membrane preparations have been used to measure Cu<sup>+</sup> transport by heterologously expressed human ATP7A (Voskoboinik et al., 2001). The  $Zn^{2+}$  level outside the vesicles was monitored using the membrane-impermeable, fluorescent Zn indicator FluoZin-1 (Gee et al., 2002). Figure 2.7 shows the ATPdependent  $Zn^{2+}$  uptake into HMA2-containing vesicles. In these experiments, as the metal is transported into the vesicles, the level of Zn-FluoZin-1 complex decreases and a consequent reduction of its fluorescence is detected. The observed decline in the uptake rate after 8 to 10 min is likely associated with enzyme inhibition by high intravesicular  $Zn^{2+}$ . Further evidence that the decrease in fluorescence is due to ATP-dependent  $Zn^{2+}$ transport by HMA2 is provided by the absence of  $Zn^{2+}$  uptake in the presence of 1.5 mM vanadate or ADP (replacing ATP in the assay medium). Similar lack of  $Zn^{2+}$  uptake was observed in experiments performed using membranes from yeast transformed with empty pYES2/CT vector. In summary, these results clearly indicate that HMA2 drives Zn<sup>2+</sup> export from the cell cytoplasm.



Figure 2.7 ATP-dependent  $\mathbb{Zn}^{2+}$  Transport by HMA2.  $\mathbb{Zn}^{2+}$  uptake into empty vector-transformed yeast ( $\triangle$ ), HMA2-expressing yeast vesicles in the absence ( $\blacktriangle$ ) and presence of vanadate ( $\bigcirc$ ) or ADP (replacing ATP; $\bigtriangledown$ ) was measured as indicated in "Materials and Methods." Results are shown as the relative change in fluorescence with respect to the maximum initial value. Values of uptake in the presence of ATP are the mean  $\pm$  SE (n = 3).

#### 2.3.2 Analysis of HMA2 Transcript Levels

The role of *HMA2* in the  $Zn^{2+}$  homeostasis in plants is also determined by its location and regulation upon plant exposure to various conditions. In a first approach to establish these characteristics, *HMA2* mRNA levels were measured in roots, leaves, stems, and flowers from 6-week-old plants and 10-d-old seedlings using semiquantitative RT-PCR. Figure 8.A shows significant *HMA2* transcript levels in all tested organs. Although slightly higher levels were detected in roots (50% higher than in leaf), the similar distribution in all organs suggests the ubiquitous expression of this ATPase.

Similar levels of *HMA2* mRNA were observed when seedlings were exposed to various metals (Fig. 8B), albeit up-regulation can be detected in the presence of  $Ag^+$  (53%) or  $Co^{2+}$  (66%). These findings can be compared with similar studies of two other Arabidopsis  $Zn^{2+}$ -ATPases. Different from *HMA2*, *HMA3* and *HMA4* mRNAs are more abundant in roots, and HMA4 transcription appears to be up-regulated in the presence of  $Zn^{2+}$  or  $Mn^{2+}$  and down-regulated in the presence of  $Cd^{2+}$  (Mills et al., 2003; Gravot et al., 2004). Thus, *HMA2*'s distinct transcript distribution pattern and regulation of expression suggest a unique role for this gene.



**Figure 2.8 HMA2 Transcript Levels.** RT-PCR (25 cycles) was used to amplify a 2,056-bp fragment of the *HMA2* mRNA. *eEF1* amplification (20 cycles) was used as a control of total RNA levels. A, *HMA2* mRNA levels in various organs of 6-week-old plants. B, *HMA2* mRNA levels in 10-d-old seedlings grown in the presence of the indicated metal.

## 2.3.3 Analysis of Zn<sup>2+</sup> Homeostasis in *hma2* Mutant Plants

Homozygous plants for a T-DNA insert in the first intron of the *HMA2* gene were isolated by screening the Salk\_034292 Arabidopsis line ((Alonso et al., 2003); Fig. 2.9). In a recent report, Hussain et al. (2004), described the isolation of homozygous plants for this mutant line and named it *hma2-4*. By performing back-crosses to wild type, these investigators verified that *hma2-4* plants segregated for a single T-DNA insert (Hussain et al., 2004). In our laboratory, we determined that full-length HMA2 transcripts were absent in these plants (Fig. 2.9C and D). *hma2-4* plants grow at a normal rate with no observable distinctive morphological phenotypes (Fig. 2.9F). Although *hma2-4* seedling roots appear 10% to 20% shorter than those of wild type, no statistically significant difference in root length could be established (not shown). Exposure of *hma2-4* plants (in soil or in agar) to  $Cd^{2+}$  or high  $Zn^{2+}$  did not reveal any growth or morphological alteration. These observations were supported by similar findings in *hma2-5* plants. This T-DNA insertion mutant was isolated by Harper and collaborators. *hma2-5* plants also lack full-length *HMA2* transcripts (Fig. 2.9E).

In spite of the absence of macroscopic changes, large alterations in  $Zn^{2+}$  homeostasis were observed in *HMA2* knockout plants. Under normal growth conditions, mutant plants show a 65% increase in whole-plant  $Zn^{2+}$  levels (Fig. 2.10A). This imbalance is also observed when plants are exposed to high  $Zn^{2+}$  concentrations (50%–130% increase). Keeping in mind that HMA2 exports  $Zn^{2+}$  from the cell cytoplasm, these results are in agreement with the location of this pump in the cell plasma membrane (Hussain, 2004).



**Figure 2.9 Isolation of** *hma2-4* **Mutants.** A, Schematic map of *HMA2* gene carrying a single copy of the T-DNA insert. Arrows indicate the annealing position of specific primers for the *HMA2* and left border of T-DNA insert. B, Screening of *hma2-4* mutants by PCR: DNA amplification for wild-type (WT), heterozygous *hma2-4* mutants (*hma2-4*, ht), and homozygous *hma2-4* mutants (*hma2-4*, hm) are shown. Bold letters indicate the primer pairs used for DNA amplification. C, Northern-blot analysis of *HMA2* mRNA levels in wild-type (WT) and homozygous *hma2-4* mutants (*hma2-4*). Equal amount of loading was verified by the staining of 18S rRNA with ethidium bromide (lower image). D, RT-PCR analysis of *HMA2* mRNA levels in wild-type (WT) and homozygous *hma2-4* mutants (*hma2-4*) using primers D and E. Equal amount of cDNA in each PCR tube was verified by amplification with *eEF1* primers (lower image). E, RT-PCR analysis of *HMA2* mRNA levels in wild-type (WT) and homozygous *hma2-5* mutants (*hma2-5*) using primers D and E. Equal amount of cDNA in each PCR tube was verified by amplification with *eEF1* primers (lower image). F, Wild-type and *hma2-4* plants grown in soil for 3 weeks.

To test in vivo the HMA2 capacity of transporting  $Cd^{2+}$ , the effect of exposing wild-type and mutant plants to this metal was analyzed. In preliminary control experiments, the absence of Cd<sup>2+</sup> in plants drenched in water was confirmed. When exposed to Cd<sup>2+</sup>, *hma2-5* and *hma2-4* plants accumulate higher amounts of this metal than wild-type plants (Fig. 2.10B).  $Cd^{2+}$  accumulation mimics the increase in  $Zn^{2+}$  levels in mutant plants and is in agreement with the metal specificity determined in biochemical assays. It was also observed that wild-type plants accumulated more  $Zn^{2+}$  when exposed to  $Cd^{2+}$  (Fig. 2.10A). This is likely associated with competition of both cations for cell efflux systems, HMA2 among others. The specific competition of  $Zn^{2+}$  and  $Cd^{2+}$  for HMA2 is clearer when  $Zn^{2+}$  levels in Cd<sup>2+</sup>-exposed *hma2-5* and *hma2-4* plants are analyzed (Fig. 2.10A). In this case, a reduction (25%-30%) in the Zn<sup>2+</sup> levels is observed. These results can be interpreted in terms of a parsimonious model where  $Zn^{2+}$ homeostasis is controlled by a cell influx component (transporter), an efflux system (a metal pump, HMA2), and a component that pumps the metal into an intracellular storage compartment. Thus, removal of the efflux system (in these experiments by HMA2 knockout) would lead to an increase in the  $Zn^{2+}$  level. Alternatively, even in the absence of efflux (HMA2 knockout), removal of transport into the intracellular compartment and/or influx system (in this experiment by high  $Cd^{2+}$  competition) would lead to a reduction in the  $Zn^{2+}$  total level.



Figure 2.10  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Fe}^{2+}$  Levels in Wild-type, *hma2-5*, and *hma2-4* Plants. A,  $\text{Zn}^{2+}$  levels in wild-type (white bars), *hma2-5* (light gray bars), and *hma2-4* (dark gray bars) plants drenched in tap water (Water), 0.5 mM ZnCl<sub>2</sub> (Zn), or 0.125 mM CdCl<sub>2</sub> (Cd), as indicated in "Material and Methods." B, Cd<sup>2+</sup> levels in wild-type, *hma2-5*, and *hma2-4* plants drenched in 0.125 mM CdCl<sub>2</sub>. C, Fe<sup>2+</sup> content of wild-type, *hma2-5*, and *hma2-4* plants treated as indicated above. Values are the mean  $\pm$  SE (*n* = 3); tissue from three plants was pooled for each independent sample. Significant differences from the wild type as determined by Student's *t* test are indicated. \**P* < 0.05; \*\**P* < 0.005.

Finally, to verify that the observed effects are not the result of unspecific alterations in ionic homeostasis,  $Fe^{2+}$  levels were measured in wild-type, *hma2-5*, and

*hma2-4* plants. No significant changes in the level of this ion were detected in the mutant plants compared to wild type.

## **2.4 DISCUSSION**

Maintenance of  $Zn^{2+}$  homeostasis is required for normal plant physiology. This homeostasis is achieved by the specific and coordinated action of numerous secondary and primary transporters. Among these,  $Zn^{2+}$ -transporting P-type ATPases appear as likely key players considering their capacity for contraelectrochemical gradient transport and their unique presence in plants. Here we present experimental evidence for a determinant role of one of these enzymes, HMA2, in plant  $Zn^{2+}$  homeostasis.

## 2.4.1 HMA2 Biochemical Characteristics and Their Physiological Implications

HMA2 behaves as a classic P-type ATPase. It forms a phosphorylated intermediate in the presence of ATP and the outwardly transported metal and it is inhibited by vanadate. Similarly, HMA2 presents characteristics that are unique to P<sub>IB</sub>type ATPases. It is activated by several (similar) metals and requires Cys for full activity. Analysis of the enzyme metal dependence indicates that, in addition to Zn<sup>2+</sup>, its likely physiological substrate, HMA2 is also activated by Cd<sup>2+</sup>, Pb<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup>, and Cu<sup>2+</sup>. This broad metal selectivity is common to other Zn-ATPases. For instance, HMA4 confers Zn<sup>2+</sup> and Cd<sup>2+</sup> resistance when expressed in *E. coli* and yeast, respectively (Mills et al., 2003), while HMA3-expressing  $\Delta ycfT$  yeast cells present Cd<sup>2+</sup> and Pb<sup>2+</sup> tolerance (Gravot et al., 2004). A prokaryote HMA2 homolog, *E. coli* ZntA, is also activated by these divalent heavy metals (Okkeri and Haltia, 1999; Sharma et al., 2000). However, HMA2 and ZntA relative activities in the presence of various metals are different. ZntA appears activated by Pb<sup>2+</sup>  $\geq$ Zn<sup>2+</sup> > Cd<sup>2+</sup> > Ni<sup>2+</sup> = Cu<sup>2+</sup> (compare with Fig. 2.3). The multiselectivity of these enzymes is likely associated with the similar ionic radius, Lewis characteristics, and/or  $K_{eq}$  for the corresponding Cys complexes of these metals. On the other hand, the relative differences in activation patterns might be associated with small structural differences due to variations in nonmetal-coordinating amino acids located close to metal-binding sites (Argüello, 2003; Sharma et al., 2000). These molecular characteristics have direct physiological effects since, in vivo, HMA2 does transport the nonphysiological substrates as evidenced by higher Cd<sup>2+</sup> levels and the competition of Zn<sup>2+</sup> and Cd<sup>2+</sup> observed in *hma2-5* and *hma2-4* plants exposed to these metals.

HMA2 interacts with metals with particularly high affinity, approximately three orders of magnitude higher than those observed in ZntA (Sharma et al., 2000). This higher affinity for Zn<sup>2+</sup> and other substrates might lead to lower cytoplasmic levels of these metals in plants. Although it is possible that this is based on a tighter metal coordination, it appears more likely that the high metal affinity is originated in the preference of HMA2 to remain in its E1 conformation. In this case, a higher apparent affinity for ligands (ATP and metals) that bind this form would be observed. Correspondingly, lower apparent affinities of those ligands binding E2 should be detected. This would explain the relatively high vanadate IC<sub>50</sub> showed by HMA2. It is also interesting that HMA2 requires the presence of Cys in the assay medium for maximum activity as E. coli ZntA or Archaeoglobus fulgidus CopA (Cu<sup>+</sup>-ATPase) do (Sharma et al., 2000; Mandal et al., 2002). Experiments with CopA suggest that Cys is not transported by these enzymes but is rather required for substrate delivery to the transmembrane transport sites (Y. Yang, A.K. Mandal, and J.M. Argüello, unpublished data). In plants, it might not be Cys but a similar complexing or chaperone molecule that

delivers the metal to the enzyme. Soluble metal chaperones have been identified in plants (Himelblau et al., 1998).

#### 2.4.2 Physiological Role of HMA2

The increase in  $Zn^{2+}$  and  $Cd^{2+}$  levels in *hma2* plants indicates that the enzyme has a key role in maintaining metal homeostasis. Moreover, these phenomena appear as the predictable consequence of HMA2 driving the export of metals from the cytoplasm and being located in the plasma membrane rather than in an intracellular organelle. Supporting this rationale, it can be considered that, if HMA2 would transport extracellular ions into the cytoplasm, a different phenotype (reduced metal levels) would be observed. Moreover, our results correlate with the recent studies involving transgenic expression of E. coli ZntA in Arabidopsis (Lee et al., 2003). ZntA appears to be targeted to the plasma membrane of Arabidopsis protoplasts; consequently, constitutive expression of this  $Zn^{2+}$ -ATPase leads to the reduction of  $Zn^{2+}$  total levels in plants. However, in this analysis we should also consider the phenotypes observed in *hma2*, hma4, and hma2 hma4 double mutants (Hussain et al., 2004). It was reported that shoots from the *hma2-2* mutant (in the Wassilewskija ecotype background) had  $Zn^{2+}$  levels similar to wild-type plants when grown in agar in the absence or presence of 10  $\mu$ M Zn<sup>2+</sup>. Although the apparent lack of phenotype might be due to the different mutant or ecotype tested, we think that more likely the differences might be attributable to the distinct experimental conditions. In our studies, metal levels were measured in plants grown in soil, receiving an exposure to higher metal levels (0.5 mM  $Zn^{2+}$ ). (Hussain et al., 2004) also observed a decrease in Zn<sup>2+</sup> levels after irrigating soil-grown hma2 hma4 double mutants with water or 1 m<sub>M</sub> Zn<sup>2+</sup>. In this case, it is likely that the effect of the *hma2* mutation was masked by the *hma4* knockout since this mutation seems to prevent translocation of  $Zn^{2+}$  from roots to shoots, thus leading to a decreased metal level (Hussain et al., 2004).

The analysis of HMA2 function should also consider the tissue distribution of this protein. The expression of a reporter gene under the control of the HMA2 promoter region shows that HMA2 is likely expressed in vascular tissues (Hussain et al., 2004). On the other hand, metal exposure seems to have little effect on HMA2 transcript expression. Thus, transcript levels and reporter gene location in conjunction with the plasma membrane location of the protein and the observed direction of transport suggest that HMA2 might have a central role in  $Zn^{2+}$  uploading into the vasculature, particularly the phloem, while HMA4 might have a more predominant role in xylem uploading in roots. Assuming that apoplastic metal levels influence the kinetics of the various metal transporters and thus the intracellular metal levels, it can be hypothesized that, indirectly, these two Zn-ATPases affect the overall  $Zn^{2+}$  homeostasis in plants by controlling the loading of this metal into the vasculature.

In summary, our results indicate that HMA2 is a  $Zn^{2+}$ -transporting ATPase that drives the efflux of the metal into the extracellular compartment. Consequently, *HMA2* gene knockouts lead to increased levels of  $Zn^{2+}$ . The enzyme has high metal affinity and broad specificity, thus also controlling levels of nonphysiological heavy metals such as  $Cd^{2+}$ .

#### **2.5 MATERIALS AND METHODS**

*Plant Growth*- Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) seeds were sterilized for 1 min in 70% (v/v) ethanol followed by soaking for 5 min in 1.25% (v/v) bleach solution supplemented with 0.02% Triton X-100. After incubation at 4°C for 48 h, seedlings were grown vertically on 2% agar, Murashige and Skoog salt-base medium (Buer et al., 2000). Wild-type seedlings were exposed to various metal stress conditions by growing them in Murashige and Skoog medium supplemented with one of the following metals (mM): ZnSO<sub>4</sub> (0.5); CdCl<sub>2</sub> (0.25); CoCl<sub>2</sub> (0.25); CuSO<sub>4</sub> (0.1); AgNO<sub>3</sub> (0.1); MnCl<sub>2</sub> (0.25); NiSO<sub>4</sub> (0.25). Arabidopsis Columbia plants were grown in soil in a plant growth chamber at 22°C, 10,000 to 14,000 lux cool-white fluorescent light intensity under a 14-h day/10-h night cycle. Soil-grown plants were exposed to Zn<sup>2+</sup> and Cd<sup>2+</sup> by drenching them in either 0.5 mM ZnCl<sub>2</sub> or 0.125 mM CdCl<sub>2</sub> solutions every 5 d.

*HMA2 Cloning*- First-strand *HMA2* cDNA was obtained from Arabidopsis leaf RNA by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dT) primer. Second-strand synthesis was done by PCR using the first-strand cDNAs as templates and forward and reverse primers corresponding to the 5' and 3' ends of the *HMA2* predicted coding sequence (forward, 5'-ATGGCGTCGAAGAAGATGACC-3'; reverse, 5'-TTCAATCACAATCTCTTTCAAGGT-3'; At-genome, At4g30110; accession no. AY434728). Resulting cDNA was purified and ligated into the pBAD/TOPO vector (Invitrogen). The cDNA sequence was confirmed by automated DNA sequence analysis.

*HMA2 Expression in Yeast*- HMA2 cDNA was subcloned into the *Kpn*I and *Xho*I sites of the yeast (*Saccharomyces cerevisiae*) expression vector pYES2/CT (Invitrogen) under the control of a *GAL*-inducible promoter. This vector introduces a His<sub>6</sub> tag at the C-terminal

end of the protein. In control experiments, an HMA2 stop codon was included in the insert. The resulting protein lacking the His<sub>6</sub> tag was used to verify that the tag did not alter measured kinetic parameters. The forward primer used for *HMA2* cDNA amplification was designed to include a yeast consensus sequence (AATA) upstream of the initiation codon, as suggested by the vector supplier. Yeast strain INVSc1 MAT<sub> $\alpha$ </sub> *his3* $\Delta$ *1 leu2 trp1-289 ura3-52* (Invitrogen) was transformed with the HMA2-pYES2/CT or the empty pYES2/CT vector by the lithium acetate method (Ito et al., 1983), and uracil-based selection was used to screen for transformants. Yeast cells were grown overnight at 30°C in synthetic dextrose medium without uracil (6.7 g L<sup>-1</sup>, yeast nitrogen base, 1.92 g L<sup>-1</sup> yeast synthetic dropout media without uracil [Sigma, St. Louis]) supplemented with 20 g L<sup>-1</sup> Glc. To induce HMA2 expression, cells were diluted to OD<sub>600</sub> = 0.4 with the same media but containing 20 g L<sup>-1</sup> Gal instead of Glc and grown for 8 h.

*Yeast Membrane Preparation*- Membrane preparation from yeast cells was done as previously described with minor modifications (Voskoboinik, 2001). Briefly, cells were suspended in 10 mM Tris (pH 7.4), 250 mM Sucrose, 10 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g mL<sup>-1</sup> leupeptin, and 1  $\mu$ g mL<sup>-1</sup> aprotinin. Cells were disrupted in a bead beater (BioSpec, Bartlesville, OK; 4 x 30 s homogenization with 30-s intervals) and the homogenate was centrifuged at 10,000g for 20 min. The supernatant was collected and centrifuged at 110,000g for 60 min. The resulting pellet was resuspended in the buffer described above except that it contained 0.2 mM ascorbic acid. All procedures were performed at 0°C to 4°C. The membrane preparations (7–10 mg protein mL<sup>-1</sup>) were stored at –80°C. Protein was measured in accordance with (Bradford,
1976), using bovine serum albumin as a standard. SDS-PAGE was carried out in 10% acrylamide gels (Laemmli, 1970). Protein bands were observed by staining the gels with Coomassie Brilliant Blue. Heterologous protein was detected by electroblotting the gels onto nitrocellulose membranes and immunostaining with anti-His<sub>6</sub> rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-rabbit IgG-horseradish peroxidase-linked monoclonal antibody (Santa Cruz Biotechnology).

*ATPase Assays*- The ATPase assay mixture contained 50 mM Tris, pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM ATP, 20 mM Cys, 1 mM dithiothreitol (DTT), 0.5 mg mL<sup>-1</sup> saponin, 1  $\mu$ M ZnCl<sub>2</sub> (or the metal indicated in the figures), and 40  $\mu$ g mL<sup>-1</sup> protein (membrane preparation). In different experiments, these reagents were independently varied as indicated in the corresponding figures. ATPase activity was measured for 15 min at 30°C. Released inorganic phosphate was colorimetrically determined (Lanzetta et al., 1979). Background activity measured in the absence of transition metals or in membranes from empty vectortransformed yeast was less than 20% to 30% of the Zn<sup>2+</sup>- or Cd<sup>2+</sup>-stimulated activity present in HMA2-containing membranes. This background was subtracted from the activity measured in the presence of metals.

*Phosphorylation Assays*- Enzyme phosphorylation by ATP was carried out at 0°C in a medium containing 50 mM Tris, pH 7.5, 0.5 mg mL<sup>-1</sup> saponin, 1 mM MgCl<sub>2</sub>, 5  $\mu$ M [ $\gamma$ -<sup>32</sup>P] ATP (MP Biomedical, Irvine, CA), 0.04 mM EGTA, 20 mM Cys, 20% dimethyl sulfoxide, 100  $\mu$ g mL<sup>-1</sup> protein (membrane preparation), and 2.5  $\mu$ M metal as indicated in Figure 2.6. Vanadate inhibition was measured by including 1.5 mM Na<sub>3</sub>VO<sub>4</sub> in the assay medium. The reaction was initiated by the addition of [ $\gamma$ -<sup>32</sup>P] ATP. After 1 min incubation, phosphorylation was stopped with five volumes of ice-cold 10% TCA and 1 mM inorganic phosphate. In initial experiments, samples were centrifuged at 14,000g for 10 min, resuspended in acidic SDS-PAGE loading buffer (5 mM Tris-PO<sub>4</sub>, pH 5.8, 6.7 M urea, 0.4 M DTT, 5% SDS, and 0.014% bromphenol blue), and resolved by SDS-PAGE in 8% acidic gels (Sarkadi et al., 1986). The gels were dried and radioactivity was monitored in a phosphoimager. In subsequent experiments, the samples were filtered through nitrocellulose 0.45- $\mu$ m filters (Millipore, Billerica, MA), washed five times with acid-stopping solution, and radioactivity was measured in a scintillation counter. Background phosphorylation measured in the absence of transition metals or in membranes from empty vector-transformed yeast was less than 5% to 10% of the Zn<sup>2+</sup>- or Cd<sup>2+</sup>-stimulated activity present in HMA2-containing membranes. This background was subtracted from phosphorylation measured in the presence of metals.

 $Zn^{2+}$  *Transport Assays*- The assay mixture contained 50 mM Tris, pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM ATP, 5 mM Cys, 1  $\mu$ M ZnCl<sub>2</sub>, 5  $\mu$ M FluoZin-1 (Molecular Probes, Eugene, OR), and 100  $\mu$ g mL<sup>-1</sup> protein (membrane preparation). Vanadate inhibition was tested by including 1.5 mM Na<sub>3</sub>VO<sub>4</sub> in the assay medium. Metal uptake was initiated by the addition of ATP. Zn-FluoZin-1 was excited at 495 nm and emission measured at 520 nm. The indicator showed a linear fluorescent response in the 0.25- to 5- $\mu$ M Zn<sup>2+</sup> range. None of the reagents in the assay media produced detectable fluorescence quenching. Determinations were performed at 25°C.

*mRNA Level Analysis*- Total RNA was isolated using the RNeasy-Midi kit (Qiagen, Valencia, CA) from Arabidopsis 10-d-old seedlings grown either in agar plates containing Murashige and Skoog or Murashige and Skoog supplemented with various metals (see above) and from soil-grown 6-week-old plants (roots, leaves, stems, and flowers). cDNA

synthesis was performed with SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) as primer. The PCR amplification was performed with a cDNA aliquot and gene-specific primers for *HMA2* (forward, 5'-TGCTGTACATCGGAGGTTCCGT-3' and reverse, 5'-CACTGAGCAACAACATGCTATTAAGG-3') and the ubiquitous  $eEF1\alpha$  (forward, 5'-AGGAGCCCAAGTTTTTGAAGA-3' and reverse, 5'-TTCTTCACTGCAGCCTTGGT-3'). Samples were taken after each cycle and amplified bands quantified in agarose gels to verify that saturation has not been reached. Consistent results were obtained in two fully independent experiments.

*Northern-Blot Analysis*- Total RNA was extracted as indicated and denatured by incubating 15 min at 55°C. Samples were separated by denaturing agarose gel electrophoresis and transferred to Immobilon nylon membranes (Millipore; (Sambrook J, 1989)). Equal loading of RNA in each lane was confirmed by ethidium bromide staining of 18S rRNA. Probes were prepared by amplification of a 1,420-bp DNA fragment that is complementary to the cDNA fragment between 49 and 1,469 bp. Probes were labeled with [ $\alpha$ -<sup>32</sup>P]dATP (Amersham Biosciences, Piscataway, NJ) by random hexamer primers. After hybridization at 65°C, the nylon membranes were washed twice for 15 min at 65°C in a low-stringency wash solution (2x SSC/0.1% SDS). Radiolabeled bands were detected by autoradiography.

*Metal Content Analysis*- Determinations were performed using whole-plant samples (approximately 250 mg) from 4-week-old wild-type and mutant plants. Three plants were pooled for each independent determination. Samples were washed with distilled water, drained, and acid digested at 80°C for 4 h and then overnight at room temperature with 7 mL 4.5 N HNO<sub>3</sub>. After digestion, 0.5 mL 30%  $H_2O_2$  were added and samples diluted with

water to 10-mL final volume. Metal (Zn, Fe, and Cd) contents were measured by atomic absorption spectroscopy (AAnalyst 300; Perkin-Elmer, Foster City, CA).

Insertional Mutant Isolation- The Salk 034393 Arabidopsis line carrying a T-DNA insert approximately 140 bp from the start of the first intron in the HMA2 gene (Alonso et al., 2003) was obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous mutants (referred to as hma2-4 in this thesis) were identified by PCR screening using genomic DNA as template and separated combinations of a primer sitting in the left border of the T-DNA insert (5'-GCGTGGACCGCTTGCTGCAACT-3'; primer С primers in Fig. 2.9) and *HMA2*-specific (forward, 5'-CGACAACGTTATCATTCATACCCATC-3' 5'and reverse. AATTGGTTTCCCGGTTACCCTCAC-3'; primers A and B, respectively, in Fig. 2.9). The absence of a full-length HMA2 transcript was confirmed by RT-PCR (forward, 5'-TGCTGTACATCGGAGGTTCCGT-3' 5'and reverse, CACTGAGCAACAACATGCTATTAAGG-3'; primers D and E in Fig. 2.9) and northern-blot analysis. The *hma2-5* mutant was obtained from Jeffrey Harper's laboratory (The Scripps Research Institute, La Jolla, CA). This mutant is homozygous for a T-DNA insertion in the fourth exon of the HMA2 gene (404 B12; Syngenta, San Diego). The absence of a full-length HMA2 transcript in hma2-5 mutant plants was confirmed by RT-PCR analysis in our laboratory.

*Material Distribution*- Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requester. *Sequence Analysis*- Sequences were aligned using the LaserGene software package (DNASTAR, Madison, WI). HMA2 membrane topology was obtained using the TMHMM 2.0 on-line server for prediction of transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM).

*Data Analysis*- Curves of ATPase activity versus metal were fit to  $v = V_{\text{max}} L/(L + K_{1/2})$ , where *L* is the concentration of variable ligand. ATPase activity versus vanadate curves were fit to  $v = (V_{\text{max}} - V_{\text{min}})/[1 + (I/K_{1/2})] + V_{\text{min}}$ , where *I* is the concentration of inhibitor,  $K_{1/2}$  is the inhibitor concentration that produces one-half the inhibitory effect, and  $V_{\text{min}}$  is the activity at maximum inhibition. Data analysis was done using the KaleidaGraph software (Synergy, Reading, PA). The reported SEs for  $V_{\text{max}}$  and  $K_{\text{m}}$  are asymptotic SEs reported by the fitting program.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY434728.

The most exciting phrase to hear in science, the one that heralds new discoveries, is not

"Eureka!" (I found it!) but "that's funny..."

Isaac Asimov

# A Novel Regulatory Metal Binding Domain Is Present in the C Terminus of Arabidopsis Zn<sup>2+</sup>-ATPase HMA2

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### **3.1 ABSTRACT**

HMA2 is a Zn<sup>+2</sup>-ATPase from Arabidopsis thaliana. It contributes to the maintenance of metal homeostasis in cells by driving Zn<sup>+2</sup> efflux. Distinct from P<sub>1B</sub>-type ATPases. plant Zn<sup>+2</sup>-ATPases have relatively long C-terminal sequences rich in Cys and His. In this report, we show that these sequences are metal binding domains with likely relevant functions. Removal of the 244 amino acid C-terminus of HMA2 leads to a 43% reduction in enzyme turnover without significant effect on the  $Zn^{+2} K_{1/2}$  for enzyme activation. Characterization of the isolated HMA2 C-terminus showed that this fragment binds three  $Zn^{+2}$  ions with high affinity (K<sub>d</sub> = 16 ± 3 nM). Circular dichroism spectral analysis indicated the presence of 8% alpha helix, 45% beta sheet and 48% random coil in the C-terminal peptide with noticeable structural changes upon metal binding (8% alpha helix, 39% beta sheet and 52% random coil in the presence of  $Zn^{+2}$ ). Studies of the Zn complexes formed using Zn K-edge XAS and chemical modification of His and Cys residues show that His coordination plays major role in forming the Zn complexes, while Cys coordination is much less important. Zn K-edge XAS of Zn-C-MBD in the presence of one equivalent of Zn<sup>+2</sup>, shows that the average Zn complex formed is composed of three His and one Cys residues. Upon the addition of two extra Zn<sup>+2</sup> ions per C-MBD, these appear coordinated primarily by His residues thus, suggesting that the three Zn binding domains might not be identical. Modification of His residues with diethyl pyrocarbonate (DEPC) completely inhibited  $Zn^{+2}$  binding to the C-terminus, pointing out the importance of His residues in  $Zn^{+2}$  coordination. In contrast, alkylation of Cys with iodoacetic acid (IAA) did not prevent Zn<sup>+2</sup> binding to the HMA2 C-terminus. Zn K-edge XAS of the Cys-alkylated protein was consistent with (N/O)<sub>4</sub> coordination of the Zn site,

with three of those ligands fitting for His residues. In summary, plant  $Zn^{+2}$ -ATPases contain novel metal binding domains in their cytoplasmic C-terminus. Structurally distinct from the well characterized N-terminal metal binding domains present in most  $P_{1B}$ -type ATPases, they also appear to regulate enzyme turnover rate.

#### **3.2 INTRODUCTION**

 $P_{1B}$ -type ATPases, a subfamily of P-type ATPases, transport heavy metals (Ag<sup>+</sup>, Cu<sup>+</sup>, Cu<sup>+2</sup>, Zn<sup>+2</sup>, Cd<sup>+2</sup>, Pb<sup>+2</sup>, Co<sup>+2</sup>) across biological membranes (Argüello, 2003; Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995). These enzymes play critical roles in maintaining heavy metal homeostasis in organisms ranging from bacteria to humans (Bull and Cox, 1994; Lutsenko and Petris, 2003; Petris et al., 1996; Rensing et al., 1999). Plant genomes appear to contain multiple [8-9] genes encoding P<sub>1B</sub>-ATPases with various distinct metal selectivities (Zn<sup>+2</sup>-ATPases, Cu<sup>+</sup>-ATPases, and others with metal dependence is still to be determined) (Argüello, 2003; Axelsen and Palmgren, 2001; Williams and Mills, 2005). Distinctly, only two Cu<sup>+</sup>-ATPase isoforms are found in other eukaryotes (Argüello, 2003; Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995). We recently characterized the functional role of Arabidopsis thaliana HMA2 (Eren and Argüello, 2004). This Zn<sup>+2</sup>-ATPase drives the efflux of metals out of the cell and is activated by  $Zn^{+2}$  and  $Cd^{+2}$  with quite low apparent affinities (0.1-0.2  $\mu$ M). Analysis of A. thaliana hma2 knock-out mutants revealed a significant increase in whole plant Zn<sup>+2</sup> and Cd<sup>+2</sup> levels (Eren and Argüello, 2004). This observation along with the plasma membrane localization and strong expression in the plant vasculature suggests that HMA2 is responsible for Zn<sup>+2</sup> uploading into the phloem (Eren and Argüello, 2004; Hussain et al., 2004).

 $P_{1B}$ -type ATPases have 6-8 transmembrane fragments responsible for metal translocation and a large cytoplasmic loop involved in ATP binding and hydrolysis (Argüello, 2003; Axelsen and Palmgren, 1998; Lutsenko and Kaplan, 1995). Conserved residues in transmembrane fragments H6, H7 and H8 participate in metal coordination

during transport and provide signature sequences that predict the metal selectivity of  $P_{1B}$ type ATPases (Argüello, 2003; Mandal et al., 2004). Most of these enzymes also have highly conserved N-terminal metal binding domains (N-MBDs) characterized by the CXXC sequences (Argüello, 2003; Arnesano et al., 2002; Lutsenko and Petris, 2003; Rensing et al., 1999). These Cys residues are responsible for metal coordination, and can bind both monovalent and divalent cations  $(Cu^+, Cu^{+2}, Zn^{+2}, Cd^{+2})$  (Banci et al., 2002; DiDonato et al., 1997; Gitschier et al., 1998; Harrison et al., 1999; Jensen et al., 1999; Lutsenko et al., 1997). In Cu<sup>+</sup>-ATPases, N-MBDs receive the metal from specific Cu<sup>+</sup>chaperones (Hamza et al., 1999; Huffman and O'Halloran, 2000; Larin et al., 1999; Strausak et al., 2003; Walker et al., 2002; Wernimont et al., 2000, 2004). Removal of the N-MBDs metal binding capability by truncation or mutation leads to reduced enzyme activity with small or no changes in metal affinity (Bal et al., 2001; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003; Mitra and Sharma, 2001; Voskoboinik et al., 1999, 2001). Lutsenko and coworkers have shown the Cu<sup>+</sup> dependent interaction of Wilson's disease protein N-MBDs with the large ATP binding cytoplasmic loop (Tsivkovskii et al., 2001). In our laboratory we have observed that N-MBDs of Archaeoglobus fulgidus CopA, a Cu<sup>+</sup>-ATPase, and CopB, a Cu<sup>+2</sup>-ATPase with a His rich N-MBD, control the turnover rate of these enzymes but do not affect metal binding to the transport site (Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Specifically, the rate limiting conformational change associated with metal release/dephosphorylation is affected by metal binding to N-MBDs (Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Thus, N-MBDs, although not essential for activity, are key regulators of enzyme function. In addition, studies of the human Cu<sup>+</sup>-ATPases, Menkes and Wilson dDisease

proteins that contain six N-MBDs, suggest that these (or a subset of them) are required for copper-induced relocalization of these ATPases from the trans-Golgi network to the plasma membrane and a vesicular compartment, respectively (Forbes et al., 1999; Schaefer et al., 1999; Strausak et al., 1999; Petris et al., 1996).

Many bacterial Zn<sup>+2</sup>-ATPases seem to contain the typical CXXC N-MBDs (Argüello, 2003; Mitra and Sharma, 2001). It has been shown that in ZntA, Cys in the conserved GMDCXXC motif coordinate metal ions with high affinity (Banci et al., 2002; Liu et al., 2005). Similar to Cu<sup>+</sup>-ATPases N-MBDs, ZntA N-MBD is not essential for enzyme activity but truncation of this domain results in a decrease in overall rate of the enzyme without altering metal affinity (Mitra and Sharma, 2001; Liu et al., 2006). Interestingly, all eukaryote (plant)  $Zn^{+2}$ -ATPases lack the typical N-MBDs. In these, the CXXC conserved sequence is replaced by CCXSE (X=S,T,P) (except Oryza sativa HMA3 which has CCXAE). In addition, all plant  $Zn^{+2}$ -ATPases appear to have unusually long C-termini ranging from 61 amino acids in Arabidopsis halleri HMA3 to 479 amino acids in *Thalaspi caerulescens* HMA4. These contain numerous CysCys repeat sequences and His residues. These Cys and His rich fragments are uncommon among non-plant P<sub>1B</sub>type ATPases. Considering the metal ligating capability of sulfhydryl and imidazole side chains, then it is tempting to hypothesize that these might constitute C-terminal metal binding domains (C-MBDs). However, studies based on functional complementation approaches have provided conflicting results on the roles of Zn<sup>+2</sup>-ATPases putative C-MBDs. Truncation of the C-terminus His rich stretch (the last 16 amino acids of the Cterminus) of A. thaliana HMA4 impaired the enzyme ability to complement vcfl (Cd<sup>+2</sup> sensitive) and zrc1 ( $Zn^{+2}$  sensitive) yeasts in the presence of high  $Cd^{+2}$  or  $Zn^{+2}$  (Verret et al., 2005). In a different study, truncation of its whole C-terminus did not affect the capacity of *A. thaliana* HMA4 to confer  $Cd^{+2}$  resistance to the *ycf1* yeast (Mills et al., 2005). Thus, the functional role of the long cytoplasmic C-terminus of plant  $Zn^{+2}$ -ATPases has not been established.

Here, we describe the functional role of *A. thaliana* HMA2 C-MBD. The ATPase kinetics and metal dependence of truncated HMA2, lacking the cytoplasmic C-terminus fragment, was characterized. In addition, the isolated cytoplasmic C-terminus fragment was heterologously expressed and its metal binding properties were determined. Our data show that the HMA2 C-terminus contains a novel domain with multiple metal binding sites. Moreover, they indicate that metal binding to this C-MBD probably regulates the enzyme turnover rate.

#### **3.3 RESULTS**

Figure 3.1A shows the membrane topology of HMA2 based on its homology to other P<sub>1B</sub>-ATPases (Eren and Argüello, 2004). Conserved residues in H6, H7, and H8 point out the location of putative transmembrane metal binding sites responsible for metal translocation during catalysis. The site of catalytic phosphorylation (D391) in the large cytoplasmic loop is also indicated. Flanking the transmembrane region, the putative cytoplasmic metal binding domains are highlighted. The N-MBD likely extends till V76 while the C-MBD fragment characterized in this study starts at S708. Figure 3.1B shows the 244 amino acid long C-terminal fragment of A. thaliana HMA2 (C-MBD). This fragment contains 20 Cys and 20 His residues (note that there are two additional His residues that are added by the pPRIBA1). Among these, there are six CC and five HXH repeats with three of them arranged in a CCX<sub>7</sub>HXH pattern. In addition, the sequence DSGCCGXKSQQPHQHEXQ appears twice. All these distinct sequences might potentially contribute to metal binding sites. Sequence analysis of Zn<sup>+2</sup>-ATPases from various plant species including A. thaliana, T. caerulescens, O. sativa and A. halleri shows that in all these species the C-terminus is rich in Cys and His (Eren and Argüello, unpublished results). Although these C-termini do not share highly homologous sequences that point out metal binding sites, they have some conserved fragments including SSDHS/LHS/P, KKSCC, CCG/DXK, QSCHN/EK, CCRSYAK and CSHXHn (n = 3-11) that can certainly have this role.

Α.

Extracellular



MGDRGPEFELGTSSSSSVLIAEKLEGDAAGDMEAGLLPKISD KHCKPGČČGTKTQEKAMKPAKASSDHSHSGČČETKQKDNV TVVKKSČČAEPVDLGHGH<u>DSGČČGDKSQQPHQHEVQ</u>VQQ SCHNKPSGL<u>DSGČČGGKSQQPHQHELQ</u>QSCHDKPSGLDIGT GPKHEGSSTLVNLEGDAKEELKVLVNGFCSSPADLAITSLKV KSDSHCKSNCSSRERCHHGSNČČRSYAKESCSHDHHHTRAH GVGTLKEIVIELEVDLQGDHGLSA<u>WSHPQFEK</u>

**Figure 3.1 Structural Features of HMA2 and C-MBD.** *A*, topology of HMA2. *Numbers* in *white boxes* indicate the position of transmembrane segments within the HMA2 sequence.  $C^{347}PC$ , Lys<sup>658</sup>, Asp<sup>679</sup>, and Gly<sup>681</sup> are conserved in all Zn<sup>2+</sup>-ATPases (3). *Black boxes* represent putative metal binding domains. Ser<sup>708</sup> is the starting amino acid of the C-MBD fragment used in this study. *B*, C-MBD sequence. The *arrows* indicate the beginning and ending of the C-MBD. Flanking sequences are inserted by the expression vector. His residues in HXH repeats are shown in *bold* and Cys-Cys dipeptides are indicated with *asterisks*. The duplicated sequences DSGCCGXKSQQPHQHEXQ are *underlined*. The Strep tag sequence is *boxed*.

To explore the functional role and metal binding characteristics of HMA2 C-MBD, various protein constructs were designed (Fig. 3.2A). The C-MBD 244 amino acid fragment of HMA2 was expressed in a soluble form and affinity purified (Fig. 3.2B, lanes 3 and 5). A small fraction < 20% of the C-MBD was consistently observed as a  $\beta$ -mercaptoethanol resistant dimer. HMA2 lacking the C-MBD ( $\Delta$ C-HMA2) or both the C-MBD and N-terminus ends ( $\Delta$ NC-HMA2) were expressed in yeast where they were targeted to membrane fractions (Fig. 2C). Truncated proteins expressed at levels different from wild type HMA2 (relative expression: HMA2 = 1;  $\Delta$ C-HMA2 = 1.25 ± 0.14 and  $\Delta$ N,C-HMA2 = 1.36 ± 0.13). These differences were later considered in ATPase activity determinations.



Figure 3.2 Expression of HMA2 Proteins and Purification of C-MBD. *A*, schematic representation of HMA2 constructs used in this study. *Black blocks* represent the transmembrane fragments; *white blocks*, extramembranous regions; and *gray block*, the C-MBD. Conserved Asp<sup>391</sup> is represented with a *line*. The position of starting and ending amino acids in each construct is indicated. *B*, expression and purification of HMA2 C-MBD. Bacterial cell lysate from empty pPRIBA1 transformed cells (*lanes 1* and *4*); cell lysate from induced C-MBD-pPRIBA1-transformed cells (*lane 2*); and

purified C-MBD (*lanes 3* and 5). *Lanes 1–3*, Coomassie Brilliant Blue-stained gel; *lanes 3* and 4 blot immunostained with Strep-Tactin horseradish peroxidase antibody. *C*, expression of HMA2 Strep-tagged proteins. Membrane preparations from yeast transformed with HMA2-pYES2/Strep (*lane 1*);  $\Delta$ C-HMA2-pYES2/Strep (*lane 2*);  $\Delta$  NC-HMA2-pYES2/Strep (*lane 3*); and from untransformed yeast (*lane 4*). *Lanes 1–4*, blot immunostained with Strep-Tactin horseradish peroxidase antibody. *D*, relative expression levels of HMA2,  $\Delta$ C-HMA2, and  $\Delta$ N,C-HMA2. Dot immunoblot of HMA2 (*1*),  $\Delta$ C-HMA2 (*2*), and  $\Delta$ N,C-HMA2 (*3*) membrane preparations at two different dilutions.

#### **3.3.1 Effect of C-MBD truncation on HMA2 ATPase activities**

Toward characterizing the C-MBD region, the first question to be addressed was whether it plays a functional role. Because of the large number of the residues that might participate in metal coordination and the present uncertainties on which ones might play this role, rather than a mutagenesis approach, characterization of truncated HMA2 was the chosen strategy. Removal of the HMA2 C-MBD led to significant decrease of the enzyme turnover rate (Fig. 3.3A and 3.3B). The role of C-MBD appears independent of the presence of the N-terminus of the enzyme since no significant kinetic differences were detected among  $\Delta$ C-HMA2 and  $\Delta$ NC-HMA2 proteins. Both truncated proteins exhibit similar V<sub>max</sub> and metal dependence. Interestingly, truncation of HMA2 C-MBD led to a small but detectable reduction in the apparent affinity of the enzyme for Zn<sup>+2</sup> or Cd<sup>+2</sup>. Keeping in mind that V<sub>max</sub> is measured at saturating metal concentrations, it is clear that the small changes in activating metal affinity do not explain the reduction of V<sub>max</sub>.



Figure 3.3 ATPase Activity of HMA2 Proteins. *A*,  $Zn^{2+}$ -dependent ATPase activities of HMA2 proteins. Data were fitted using the following parameters for  $Zn^{2+}$ : HMA2 (•)  $K_{1/2} = 0.13 \pm 0.03 \mu$ M,  $V_{max} = 100 \pm 6\%$ ;  $\Delta$ C-HMA2 (•)  $K_{1/2} = 0.29 \pm 0.06 \mu$ M,  $V_{max} = 56.6 \pm 4.3\%$ ;  $\Delta$ NC-HMA2 (•)  $K_{1/2} = 0.33 \pm 0.11 \mu$ M,  $V_{max} = 44.2 \pm 5.6\%$ . 100% = 1.43  $\mu$ mol·mg<sup>-1</sup>·hr<sup>-1</sup>. Relative activity values for  $\Delta$ C-HMA2 and  $\Delta$ NC-HMA2 (•)  $K_{1/2} = 0.037 \pm 0.008 \mu$ M,  $V_{max} = 100 \pm 4.5\%$ ;  $\Delta$ C-HMA2 (•)  $K_{1/2} = 0.066 \pm 0.018 \mu$ M,  $V_{max} = 50.1 \pm 1.8\%$ ;  $\Delta$ NC-MA2 (•)  $K_{1/2} = 0.049 \pm 0.007 \mu$ M,  $V_{max} = 40.6 \pm 2.9\%$ . 100% = 1.55  $\mu$ mol·mg<sup>-1</sup>·h<sup>-1</sup>. Values are the mean  $\pm$  S.E. (*n* = 3).

It is also important to point out that the removal of HMA2 C-MBD had no effect on the relative activation by  $Zn^{+2}$  and  $Cd^{+2}$  or the relative enzyme affinity for each of these metals; i.e., all three proteins had 4-6 times higher affinity for  $Cd^{+2}$  than for  $Zn^{+2}$ . This observation contributes to the idea that the removal of C-MBD affects enzyme velocity without changing metal binding to transmembrane transport sites.

#### 3.3.2 Metal Binding to C-MBD

Table 3.1 shows the determination of metal binding to C-MBD by Atomic Absorption Spectroscopy (AAS). This indicated that C-MBD indeed binds  $Zn^{+2}$  and  $Cd^{+2}$ with a stoichiometry of three metals per C-MBD molecule. It is interesting that the stoichiometry of  $Zn^{+2}$  binding is unchanged under reducing (in the presence of TCEP) or non-reducing conditions. On the contrary, binding of  $Co^{+2}$  to the C-MBD fragment was affected by the presence of TCEP suggesting a different binding site for the non activating metal.

Table 3.1 Determination of Metal Binding Stoichiometry to C-MBD

Metal	Metal bound/C-MBD <sup>a</sup>				
	C-MBD + TCEP	C-MBD			
$Zn^{+2}$	$2.93 \pm 0.23$	$3.10 \pm 0.12$			
$\mathrm{Cd}^{+2}$	$3.15 \pm 0.35$	n.a. <sup>b</sup>			
Co <sup>+2</sup>	$2.34 \pm 0.12$	$0.64 \pm 0.20$			

<sup>a</sup> The metal content of control samples were < 15% of the protein molar concentration. The reported values are averages of four independent sample preparations.

<sup>b</sup> n.a. = not analyzed

# 3.3.3 Zn<sup>+2</sup> Titrations of C-MBD

Since  $Zn^{+2}$  is spectroscopically silent, to determine the affinity of C-MBD for  $Zn^{+2}$ , we performed a competition assay with the fluorescent  $Zn^{+2}$  indicator mag-fura-2 (Walkup and Imperiali, 1997). Mag-fura-2 forms a 1:1 complex with  $Zn^{+2}$  with a K<sub>a</sub> of 5 x  $10^7$  M<sup>-1</sup> (Walkup and Imperiali, 1997). When mag-fura-2 forms a complex with the metal, there is a shift in its absorbance maximum from 366 nm to 325 nm with a substantial decrease in the molar absorptivity at 366 nm (mag-fura2  $\varepsilon_{366} = 1880 \text{ M}^{-1} \text{ cm}^{-1}$ ; mag-fura-2-Zn  $\varepsilon_{366}$  = 29900 M<sup>-1</sup> cm<sup>-1</sup>) (VanZile et al., 2000). Figure 3.4A shows the changes in mag-fura-2 spectra upon binding increasing  $Zn^{+2}$  levels in the presence of C-MBD. Fitting of mag-fura-2  $A_{366}$  vs. free  $[Zn^{+2}]$  allowed us to calculate the affinity of C-MBD for  $Zn^{+2}$  (K<sub>d</sub> = 1/K<sub>a</sub> = 15.6 ± 2.6 nM) and the apparent stoichiometry of the interaction (2.97  $\pm$  0.13 Zn<sup>+2</sup>/C-MBD) (Fig. 3.4B). This last parameter correlates with the determination of metal bound to C-MBD by atomic absorption spectroscopy under saturating metal conditions (Table 3.1). On the other hand, the observed K<sub>d</sub> value is similar to that described for other  $Zn^{+2}$  binding proteins (Guo and Giedroc, 1997; Liu et al., 2005; VanZile et al., 2000).



**Figure 3.4**  $\mathbb{Zn}^{2+}$  **Binding to C-MBD.** *A*, representative spectra of titration of 10 µM C-MBD and 20µM mag-fura-2 with increasing  $\mathbb{Zn}^{2+}$  concentrations (5–100 µM). The *arrow* shows the direction of absorbance change at 366 nm as increasing concentrations of  $\mathbb{Zn}^{2+}$  are added. *B*, determination of  $K_a$  for  $\mathbb{Zn}^{2+}$  binding, and the number of metal binding sites in C-MBD. The data were fit to  $\mu = nK_a[\mathbb{Zn}^{2+}_f]/(1 + K_a[\mathbb{Zn}^{2+}]_f)$  with  $n = 2.97 \pm 0.13$  and  $K_a = 6.4 \pm 0.9 \times 10^7 \text{ M}^{-1}$ . Values are the mean  $\pm$  S.E. (n = 3).

#### 3.3.4 Circular Dichroism Analysis of C-MBD

HMA2 C-MBD appears to play a regulatory role of enzyme activity and to bind  $Zn^{+2}$  with high affinity. Further understanding of this fragment's function requires characterization of its overall structure and description of the metal binding sites. Figure 3.5 shows the circular dichroism analysis of C-MBD. In the absence of metals the C-MBD appears to have a defined structure with a high content of beta sheets (45%) and limited alpha helices (8%). Upon  $Zn^{+2}$  binding, C-MBD undergoes detectable structural changes, 6% decrease in beta sheets, 4% increase in random coils (Fig. 3.5).



Figure 3.5 Structural Changes in C-MBD in the Presence of Metals. Circular dichroism analysis of C-MBD (—), C-MBD +  $Zn^{2+}$  (…), C-MBD +  $Co^{2+}$  (- -), and C-MBD +  $Cu^+$  (-·-·). *Inset*, secondary structure elements present in C-MBD in the absence and presence of metals indicated above. *a.h.*,  $\alpha$ -helix, *b.s.*,  $\beta$ -sheet, and *r.c.*, random coil. The values are given in percentages.

However, these structural changes are different in the presence of non activating metals,  $Co^{+2}$  or  $Cu^+$ . This correlates a differential coordination environment for these metals already evidenced by the lack of  $Co^{+2}$  binding under non-reducing conditions (Table 3.1).

#### 3.3.5 Zn K-edge XAS of Zn-C-MBD

The addition of one  $Zn^{2+}$  ion to C-MBD containing three potential binding sites would result in a distribution of  $Zn^{2+}$  ions among the three sites according to their relative affinities. If one site had a significantly higher affinity than the other two, then the data would represent the structure of that single site. Alternatively, if the relative binding constants were the same, the resulting data would represent and average of the three sites. The Zn K-edge XANES spectra (Fig. 3.6) for C-MBD with one Zn atom per peptide, one Zn atom per Cys-alkylated peptide, and 3 Zn atoms per peptide, all show a Zn edge that has an energy appropriate for Zn<sup>+2</sup> centers (energy at a normalized intensity of 0.5 =9663.7 eV), with no pre-edge transitions, as is typical for Zn<sup>+2</sup> centers. The relative intensities of the first two peaks after the edge in the XANES spectrum can be used to qualitatively assign the relative N/O versus S content of tetrahedral Zn complexes (Clark-Baldwin et al., 1998). The second of these two peaks being greater in intensity in all three samples suggests that they all contain at least 2 coordinated N/O donor ligands (Fig. 3.6).



Figure 3.6 Zinc K-edge XAS of C-MBD  $Zn^{2+}$  Complexes. Fourier-transformed (FT window) 2–12.5 Å<sup>-1</sup>, uncorrected for phase shifts, and unfiltered (backtransform window 1–4 Å) EXAFS spectrum (data shown as *circles* and fit as a *solid line*). *A*, 1 zinc

atom bound to C-MBD. The fit shown was obtained for 3 N @ 1.99 Å ( $\sigma^2 = 0.006 \text{ Å}^2$ ) + 1 S @ 2.28 Å ( $\sigma^2 = 0.007 \text{ Å}^2$ ) and had a g.o.f. value of 0.51 (all three N were fit with second shell multiple-scattering imidazole parameters). *B*, 1 zinc atom bound to Cys-alkylated C-MBD. The fit shown was obtained for 4 N/O @ 1.98 Å ( $\sigma^2 = 0.003 \text{ Å}^2$ ) and had a g.o.f. value of 0.67 (three N atoms were fit with second-shell multiple-scattering imidazole parameters). *C*, 3 zinc atoms bound to C-MBD. The fit shown was obtained for 3 N @ 1.99 Å ( $\sigma^2 = 0.003 \text{ Å}^2$ ) + 1 S @ 2.23 Å ( $\sigma^2 = 0.012 \text{ Å}^2$ ) and had a g.o.f. value of 0.77 (two N atoms were fit with second shell multiple scattering imidazole parameters).

EXAFS analysis for C-MBD peptide containing one  $Zn^{2+}$  ion (Fig. 3.6) is consistent with an average Zn site composed of a N(O)<sub>3</sub>S ligand donor-atom set (Table 3.2). The best fit for the data over the range of 1-4 Å (uncorrected for phase shifts) consists of three N- and one S-donors at distances of 1.99(1) Å and 2.28(2) Å, respectively. All three N-donors can be additionally fit as His imidazoles using imidazole multiple-scattering parameters (see supporting information). This fit, obtained with a single S-donor, had a goodness of fit (g.o.f.) value (0.51) that was markedly improved over the corresponding fit lacking the S-donor (0.84). Alternative fits for a 5coordinate species with either one or two sulfur-donors had slightly better values of g.o.f. (N<sub>4</sub>S g.o.f. = 0.48, N<sub>3</sub>S<sub>2</sub> g.o.f. = 0.50), however somewhat larger values for  $\sigma^2$  (see Supporting Information) and an increase in the  $\Delta E_o$  for the S donor(s) (see Supporting Information). Thus, these fits were judged to be inferior to the four-coordinate fits.

The indication of the presence of a  $Zn^{+2}$  coordinating sulfur atom by the XAS analysis was surprising considering the similar binding stoichiometry under reducing and non reducing conditions. To further explore this we analyzed the metal coordination environment of Cys-alkylated C-MBD. EXAFS analysis for the complex of the Cys-

alkylated C-MBD peptide with one  $Zn^{2+}$ ion is consistent with an (N/O)<sub>4</sub> (Ni – N/O = 1.98(1) Å) ligand donor-atom set with *ca*. three imidazole ligands, as determined from multiple-scattering analysis (g.o.f. = 0.67). It was not possible to incorporate an S-donor in any fit for this sample. This result is consistent with the lack of available Cys residues in this sample. Comparison of the EXAFS spectrum from the Cys-alkylated sample with the spectra obtained for the peptide with one or three  $Zn^{2+}$  ions bound (Fig. 3.6) shows marked differences with the non-alkylated sample containing one  $Zn^{2+}$  ion. However, it is quite similar to the spectrum obtained for the sample prepared with three  $Zn^{2+}$  ions. This suggests that the average site in the fully loaded peptide more closely resembles that of the Cys-alkylated protein.

Sample	N(donor)	<b>R</b> (Å)	$\sigma^{2}(x10^{-3})$ (Å <sup>2</sup> )	$\Delta E_0 (eV)$	g.o.f. **		
1Zn :1CMBD	3 N	1.98(2)	5(1)	3(1)	0.51		
	1 S	2.28	7(2)	10(4)			
1Zn : 1Cys-*	4 N	1.98(1)	3(1)	4(1)	0.67		
3Zn :1CMBD	3 N	1.99(1)	3(1)	4(2)	0.78		
	1 S	2.23(6)	11(5)	5(9)			
3Zn :1CMBD	3.7 N	2.00(1)	4(1)	5(1)	0.73		
	0.3 S	2.36(6)	3(4)	17(9)			
3Zn :1CMBD	4 N	2.00(1)	4(1)	5(1)	0.78		
* unforme to Constalling late of formers of C MDD							

Table 3.2 Best Fits for EXAFS data for Zn complexes formed with C-MBD

\* refers to Cys alkylated form of C-MBD.

\*\* g.o.f. = goodness of the fitting

EXAFS analysis for the Zn C-MBD complex with three  $Zn^{2+}$  ions (Fig. 3.6) is less definitive as to whether or not Cys coordination is involved with any of the three Zn sites. Fits for a Zn site with a (N/O)<sub>3</sub>S ligand donor set (Table 3.2) or a (N/O)<sub>4</sub> donor set (Table 3.2) with a backtransform window of 1-4 Å (uncorrected for phase shifts) are equally probable (g.o.f. = 0.77 or 0.78, respectively). Multiple-scattering parameters suggest again the presence of 2-3 imidazoles in the coordination sphere regardless of whether a Sdonor is present or not. The best fits for this sample have a much greater g.o.f. value (g.o.f. = 0.77) than did the one with a single Zn, suggesting that there may be small differences between the three sites in the protein producing an average spectrum that is not completely accounted for by a single Zn complex. The ambiguity regarding the presence or absence of S-ligation may result from the population of a mixture of sites consisting of both (N/O)<sub>4</sub> and (N/O)<sub>3</sub>S coordination. In fact, if non-integer values are used in the fit, an improved fit (g.o.f. = 0.73) (Table 3.2) is found for 3.7 (N/O)-donors and 0.3 S-donors. This is consistent with two sites that only contain N/O ligation and a third site that is similar to that seen in the sample with one Zn atom (containing 3 (N/O)donors and one S-donor). Alternatively, poor fitting to a 5-coordinate and 6-coordinate models ruled out these possibilities (see Supporting Information).

# **3.3.6 Effect of Reduction and Carboxymethylation of Cysteines on Metal Binding to C-MBD**

To better understand the putative role of C-MBD Cys in metal coordination, the number of free Cys in C-MBD in the absence and presence of the reducing agent TCEP was determined. DTNB analysis showed that under reducing conditions (100x molar excess of TCEP with respect to C-MBD), the number of free Cys was calculated to be  $20.3 \pm 0.8$  per C-MBD showing that essentially all Cys were reduced under the experimental conditions used in this study. On the other hand, in the absence of any reducing agent, C-MBD has  $4.1 \pm 0.6$  free Cys per monomer. This reduction in the number of free Cys had no significant effect in the number of Zn<sup>+2</sup> binding sites (Table

3.1). On another hand, titration of C-MBD with  $Zn^{+2}$  in the presence of mag-fura-2 under non-reducing conditions showed little change in the K<sub>d</sub> of the C-MBD-Zn<sup>+2</sup> complex (17.4 ± 1.8 nM), or in the apparent number of metal binding sites (3.6 ± 0.11) (Fig. 3.7A).



Figure 3.7 Effect of TCEP and Cysteine Carboxymethylation on  $\mathbb{Zn}^{2+}$ Binding to C-MBD. Data obtained from the spectra of titration of 10 µM C-MBD and 20 µM mag-fura-2 with increasing Zn<sup>2+</sup> concentrations (5–100 µM) was used to analyze Zn<sup>2+</sup> binding to C-MBD under non-reducing conditions (*A*) and Zn<sup>2+</sup> binding to carboxymethylated C-MBD (*B*). The data were fit to  $\mathbb{W} = nK_a[\mathbb{Zn}^{2+}]_f/(1+K_a[\mathbb{Zn}^{2+}]_f$  with n =

 $3.60 \pm 0.11$  and  $K_a = 5.7 \pm 0.5 \times 10^7 \text{ M}^{-1}$  and with  $n = 2.50 \pm 0.08$  and  $K_a = 4.5 \pm 0.5 \times 10^7 \text{ M}^{-1}$ , respectively. Values are the mean  $\pm$  S.E. (n = 3).

In an alternative approach to test the participation of Cys in Zn<sup>+2</sup> coordination, the C-MBD was carboxymethylated by treatment with IAA. Surprisingly, although this yielded  $0.6 \pm 0.2$  free Cys per C-MBD peptide, AAS analysis revealed that the modified C-MBD was still able to bind  $2.95 \pm 0.24$  Zn<sup>+2</sup> per C-MBD monomer. Similarly, the IAA treatment only slightly altered the metal binding affinity and stoichiometry of the C-MBD when determined by Zn<sup>+2</sup> titration in the presence of mag-fura-2 (K<sub>d</sub> = 22.1 ± 2.8 nM, n=  $2.5 \pm 0.08$ ) (Fig. 3.7B).

# **3.3.7 Effect of Histidine Modification by DEPC on Metal Binding to C-MBD**

To verify their participation in  $Zn^{+2}$  coordination by C-MBD, His were modified by incubation with DEPC. The number of modified His was spectrophotometrically determined (23.0 ± 0.2 per C-MBD molecule) showing that essentially all the His in the cloned fragment reacted with the probe. The titration spectra of DEPC modified C-MBD with  $Zn^{+2}$  in the presence of mag-fura-2 showed no  $Zn^{+2}$  binding to the protein and appear similar to that obtained in the absence of C-MBD (Fig. 3.8A and 3.8B). These results support the participation of His in  $Zn^{+2}$  coordination during binding by C-MBD.



Figure 3.8 Effect of DEPC Modification of Histidines on  $Zn^{2+}$  Binding to C-MBD. *A*, representative spectra of titration of 10 µM DEPC modified C-MBD and 20 µM mag-fura-2 with  $Zn^{2+}$ . The *arrow* shows the direction of absorbance change at 366 nm as increasing concentrations of  $Zn^{2+}$  are added. *B*, representative spectra of titration of 20 µM mag-fura-2 with  $Zn^{2+}$  in the absence of C-MBD.

#### **3.4 DISCUSSION**

The key physiological roles of plant Zn<sup>+2</sup>-ATPases (Eren and Argüello, 2004; Gravot et al., 2004; Hussain et al., 2004; Mills et al., 2005; Verret et al., 2004) likely requires fine regulation of their turnover, location, and interaction with other proteins. Analysis of their sequences reveals the presence of interesting C- and N-termini that might play regulatory roles as it is the case of N-MBDs in Cu<sup>+</sup>-ATPases (Mana-Capelli et al., 2003; Mandal and Argüello, 2003; Lutsenko et al., 1997; Lutsenko and Petris, 2002 Voskoboinik et al., 1999). In particular, the relatively long C-termini have generated attention because they are uniquely associated to eukaryote Zn<sup>2+</sup>-ATPases (Mills et al., 2005; Verret et al., 2005; Williams and Mills, 2005). However, no particular metal binding sites are self-evident in these domains and functional complementation studies have not shown a definite role for them (Mills et al., 2005; Verret et al., 2005). Toward understanding the function of these C-termini, we used HMA2 C-terminus as a model. We investigated its metal binding capabilities and role in the enzyme ATPase activity. Results presented here support the idea of a specific role of this domain controlling the enzyme function.

#### **3.4.1 The functional role of HMA2 C-MBD**

Analysis of the  $Zn^{2+}$  dependent ATPase activity of HMA2,  $\Delta$ C-HMA2 and  $\Delta$ NC-HMA2 proteins, shows that the C-MBD is required for maximum enzyme turnover rate; however, the C-MBD does not appear to influence the interaction of metal with transport sites. This is similar to the observed roles of N-MBDs in Cu<sup>+</sup>-ATPases (Mandal and Argüello, 2003), Cu<sup>+2</sup>-ATPases (Mana-Capelli et al., 2003) and Zn<sup>2+</sup>-ATPases (Mitra and Sharma, 2001). Moreover, it is reminiscent of the regulation by various N- and C-

terminal cytoplasmic domains observed in many P<sub>2</sub>-type ATPases (Baekgaard et al., 2005; Cornelius and Mahmmoud, 2003, Rimessi et al., 2005). Then, it can be postulated that C-MBDs control the enzyme rate-limiting step as shown for Cu<sup>+</sup> and Cu<sup>2+</sup>-ATPases (Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Although this appears as a parsimonious mechanism, we cannot disregard that a non-rate limiting step in the wild type enzyme becomes determinant of V<sub>max</sub> upon truncation of the C-NMBD. Independent of the kinetic effects, circular dichroism determinations show that Zn binding leads to conformational changes in the C-MBD. This might affect a putative interaction of the C-MBD with either of the cytoplasmic A, P, and N domains involved in different aspects of ATP hydrolysis and energy transduction in P-type ATPases (Toyoshima and Inesi, 2004; Sazinsky et al., 2006a, 2006b). These domains undergo key conformational transitions during the catalytic cycle and their rates would likely be affected by changes in domaindomain interactions. These interactions have been proposed for the N-MBDs and ATP binding domains of the Wilson's disease protein (Tsivkovskii et al., 2001). However, it can also be argued that the changes of V<sub>max</sub> might be unrelated to metal binding to the C-MBD and that our observations can be an unspecific conformational effect of C-MBD truncation. However, the absence of significant changes in metal apparent affinities for ATPases activities suggests that this is not the case. Further experiments are needed to test the validity of a hypothetical regulatory mechanism based in metal-dependent cytoplasmic domain interactions.

HMA2, as other plants Zn<sup>2+</sup>-ATPases, also contains a singular N-MBD where the typical CXXC sequence present in Cu<sup>+</sup>-ATPases is replaced by CCXSE (Argüello, 2003). This domain also appears to have a regulatory role since N-terminus truncated

HMA2 has a 50% reduced turnover (Eren and Argüello unpublished results). Then, it is interesting that also  $\Delta$ C-HMA2 and  $\Delta$ NC-HMA2 show approximately a 50% reduction in turnover rate. This suggests a mechanism where both, C and N-termini, participate in a coordinated regulation. In this case, the lack of either component would lead to a reduced turnover.

Enzymatic analysis indicates that the C-MBD does not control the enzyme selectivity since  $Zn^{2+}$  and  $Cd^{2+}$  activate HMA2 and  $\Delta$ C-HMA2 with similar relative affinities. Nevertheless, the C-MBD stoichiometrycally binds three  $Zn^{2+}$  at specific sites. In this direction, the interaction with non-activating metals ( $Co^{2+}$ ,  $Cu^{2+}$ ) appears to be through different residues and to lead to alternative conformational C-MBD variants. In addition, HMA2 C-MBD binds  $Zn^{2+}$  with quite high apparent affinity. However, this likely is the product of a very low off-rate in the metal-C-MBD interaction since in our experiments the on-rate is diffusionally controlled.

#### **3.4.2 The structure of HMA2 C-MBD**

Analysis of plant  $Zn^{2+}$ -ATPase C-terminus sequences shows the presence of highly homologous short fragments and numerous residues that might participate in metal coordination (see above). However, because of the various lengths of these C-termini and lack of overall homology, metal binding sites could not be uncovered by simple comparison of linear sequences. On the other hand, experimental structural analysis of HMA2 C-MBD revealed significant information. On one hand, beta sheets appear as the predominantly secondary structure in the domain and this was specifically influenced by the presence of  $Zn^{2+}$ . On the other, the three  $Zn^{+2}$  binding sites appear structurally similar and constituted by His and probably Cys residues.

The nature of the  $Zn^{2+}$  binding sites was studied by Zn K-edge XAS and chemical modification approaches. Surprisingly, these studies establish that His residues play a key role in the formation of the Zn sites, but Cvs residues do not. Analysis of  $Zn^{+2}$  bound C-MBD with a 1:1  $Zn^{+2}$ :C-MBD stoichiometry indicates that the average Zn site features a (N/O)<sub>3</sub>S ligand donor-atom set (Table 3.2). The best fit is obtained when all three Ndonors are being additionally fit as imidazoles. Zn K-edge XAS analysis of Zn bound C-MBD with a 3:1 Zn<sup>+2</sup>:C-MBD also fits well to a site with the three imidazole-N and one thiolate ligands (Table 3.2); however, this data can also be modeled equally well with a  $(N/O)_4$  ligand set (Table 3.2) with three imidazole-N ligands. Further refinement showed that a best fit can be obtained when the data is modeled to have different Zn sites, two that only contain 4 N/O donor atoms and one that contains 3 N/O donor atoms and one sulfur donor atom. Modification of C-MBD His with DEPC inhibits the Zn<sup>+2</sup> binding supporting the involvement of His in  $Zn^{+2}$  coordination. Adenosine deaminase, carbonic anhydrase II and metallo-beta-lactamase are examples of proteins in which the  $Zn^{+2}$  is coordinated by three His ligands (Karlin and Zhu, 1997; Auld, 2001). In these, two of the coordinating His are arranged as HXH, while the third is distantly located more than 20 residues away. HMA2 C-MBD contains a number of HXH repeats with at least three  $HX_nHXH$  (n  $\ge 20$ ) arrangements. Therefore, it is tempting to hypothesize that the His in HXH repeats are involved in Zn<sup>+2</sup> coordination. The model containing two (N/O)<sub>4</sub> Zn sites and one (N/O)<sub>3</sub>S Zn site is also consistent with sequence data (Fig. 3.1B). Only one of the repeated HXH sequences in the C-terminal MBD has a proximal Cys residue (...CSHDH...) and thus could be the site preferentially occupied by the first  $Zn^{2+}$  ion bound.
The coordination with three His and one water molecule is not uncommon and has been observed in catalytic sites of several proteins including human carbonic anhydrase II and thermolysin (Auld, 2001). Also site-directed mutagenesis of the ligand binding site of the *Staphylococcus aureus*  $Zn^{+2}$  sensor CzrA, showed that two liganding His could be substituted by Asp, Glu or Asn, Gln ligand pairs while retaining a native-like tetrahedral coordination geometry (Pennela, 2006). This again supports the possible replacement of a liganding thiol with another coordinating group. A more remote alternative is the presence of buried Cys not accessible to IAA; however, this is unlikely since analysis of carboxymethylated C-MBD free thiols under denaturing conditions show the presence of <1 free thiol/C-MBD.

In summary, the C-MBD appears as a regulatory domain that is not essential for enzyme activity but it is required for full activity. Our results indicate that C-terminus of HMA2 is a novel regulatory metal binding domain with three similar  $Zn^{+2}$  binding sites. All three sites appear to contain three His ligands, with one or more of the sites being able to bind with a Cys for the fourth ligand.

### **3.5 MATERIALS AND METHODS**

Cloning and Expression of HMA2 Constructs- A. thaliana HMA2 and truncated forms of the protein were expressed containing a C-terminal Strep-tag (WSHPQFEK). HMA2 cDNA was amplified from a previously prepared HMA2-pBAD/TOPO vector (Eren and Argüello, 2004) oligonucleotides: 5'by using the 3'-AGAGGTACCAATAATGGCGTCGAAGA and AAGCTCGAGTTCAATCACAATC. Resulting cDNA was ligated into the KpnI and *XhoI* sites of pPRIBA1 vector (IBA, Göttingen, Germany). This vector introduces a Strep-tag at the C-terminal end of the protein. HMA2-Strep tag cDNA was amplified by using the oligonucleotides: 5'- AGAGGTACCAATAATGGCGTCGAAGA and 3'-GCGTTTAAACTTATTTTCGAACTGC and the HMA2-pPRIBA1 as a template. The amplicon was ligated into the KpnI and PmeI sites of the yeast expression vector pYES2/CT (Invitrogen, Carlsbad, CA). Constructs to express truncated HMA2 proteins were prepared:  $\Delta$ C-HMA2 cDNA encoding HMA2 lacking the 244 C-terminal amino acids, starting in Met1 and ending in Glu707, and  $\Delta$ NC-HMA2 encoding HMA2 lacking the N-terminal first 75 amino acids and C-terminal 244 amino acids, starting from Val76 and ending in Glu707.  $\Delta$ C-HMA2 and  $\Delta$ N,C-HMA2 constructs were amplified from HMA2-pBAD/TOPO construct by using the oligonucleotides:  $\Delta$ C-HMA2 5'-AGAGGTACCAATAATGGCGTCGAAGA 3'and GCCTCGAGCTCCCTATAACATTTGTTT;  $\Delta NC$ -HMA2 5'-GCGGTACCAAAAATGGTAACCGGAGAACCAA 3'and GCCTCGAGCTCCCTATAACATTTGTTT. Resulting cDNAs were cloned into KpnI and *XhoI* sites of HMA2-pYES2/Strep construct. Consequently, the truncated HMA2

proteins also contained a C-terminal Strep-tag. Yeast strain INVSc1 *MAT* $\alpha$  *his3* $\Delta$ *1 leu2 trp1-289 ura3-52* (Invitrogen) was transformed by electroporation of cells at 1.5 kV, 25  $\mu$ F, 200 $\Omega$ . Expression of HMA2 constructs in yeast was performed as previously described (Eren and Argüello, 2004).

*Yeast Membrane Preparation*- Membranes from yeast were prepared as previously described (Eren and Argüello, 2004; Voskoboinik et al., 2001). Protein concentrations were measured in accordance to Bradford (Bradford, 1976). SDS-PAGE was carried out in 10% acrylamide gels (Laemmli, 1970). Heterologous proteins in the membrane preparations were detected by electroblotting the gels onto nitrocellulose membranes and immunostaining with Strep-Tactin horseradish peroxidase antibody (IBA). To compare HMA2,  $\Delta$ C-HMA2 and  $\Delta$ N,C-HMA2 relative expression levels, equal amounts of each membrane preparation were subjected to a 1:2 serial dilutions. These were blotted onto a nitrocellulose membrane, immunostained as indicated, and integrated density values were quantified using AlphaImager software (Alpha Innotech Corp., San Laendro, CA).

*ATPase Assay*- Metal dependent ATPase activity determinations were performed as previously described (Eren and Argüello, 2004) in a media containing 50 mM Tris, pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM ATP, 20 mM cysteine, 1 mM dithioerithrol (DTT), 0.5 mg mL<sup>-1</sup> saponin, and 40  $\mu$ g mL<sup>-1</sup> protein (membrane preparation), at 30°C. The concentrations of ZnCl<sub>2</sub> or CdCl<sub>2</sub> were varied as indicated in the figures. ATPase activity measured in the absence of metal was always < 12.5 % of V<sub>max</sub> for all protein preparations (HMA2,  $\Delta$ C-HMA2 and  $\Delta$ N,C-HMA2). Background was subtracted from plotted values. Membrane preparation from empty vector transformed cells have no Zn-dependent ATPase activity (Eren and Argüello, 2004). Curves of ATPase activity vs. metal concentration were fit to

 $v=V_{max}L/(L+K_{1/2})$ , where L is the concentration of variable ligand. The reported standard errors for  $V_{max}$  and  $K_{1/2}$  are asymptotic standard errors reported by the fitting software (Kaleidagraph, Synergy, Reading, PA).

Cloning, Expression and Purification of HMA2 C-MBD- A cDNA coding for the last 244 amino acids of HMA2, from Ser708 to Glu951 (C-MBD) was amplified by using the 5'-GCCGGTACCTCTTCTTCTTCCTCGG 3'oligonucleotides: and AAGCTCGAGTTCAATCACAATC and HMA2-pBAD/TOPO as a template. Resulting amplicon was cloned into the KpnI and XhoI sites of the bacterial expression vector pPRIBA1 (C-MBD-pPRIBA1). This introduces a Strep-tag into the C-terminal end of the protein. E. coli BL21(DE3)pLysS cells were transformed with this vector. C-MBD expression was induced with 0.5 mM IPTG for 3 h. Cells were collected by centrifugation at 2500 x g for 5 min and resuspended in 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM TCEP. Cells were disrupted by sonication on ice (3 x 30 sec) and centrifuged at 10,000 x g for 20 min. The resulting supernatant was collected and centrifuged at 110,000 x g for 60 min. The supernatant was passed through Strep-Tactin Superflow column (IBA). The column was washed with 5 volumes of homogenization buffer and the C-MBD was eluted with 100 mM Tris, pH 8.00, 150 mM NaCl, 1 mM TCEP, and 2.5 mM desthiobiotin. The protein was concentrated to 3 mg mL<sup>-1</sup> using an Amicon Ultra-15 Centricon (Millipore, Billerica, MA) and stored in 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM TCEP at -80°C. Routine protein concentration determinations were performed in accordance to Bradford (Bradford, 1976). The accuracy of colorimetric protein measurements was confirmed by total amino acid hydrolysis (Keck Facility, Yale University, New Haven, CT). Before metal binding stoichiometry determinations the storage buffer was exchanged with 20 mM HEPES, pH 7.5, and 100 mM KCl using a Sephadex G-10 column (Sigma, St. Louis, MO). Similarly, for  $Zn^{+2}$  binding titrations the storage buffer was exchanged with 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM TCEP.

*Metal Binding Stoichiometry Determination*- Metal contents were measured by atomic absorption spectroscopy (AAS) (AAnalyst 300, Perkin-Elmer, Foster City, CA). All the buffers used in these determinations were passed through a Chelex-100 column (Sigma). 30  $\mu$ M C-MBD was incubated with 0.5-1.0 mM metal (Zn<sup>+2</sup>,Cd<sup>+2</sup> and Co<sup>+2</sup>) in the presence and absence of 1 mM TCEP at 4°C for 30 min. Excess metal was removed either by passage through a Sephadex G-10 column (Sigma) or by an Amicon Ultra-15 Centricon (Millipore), both methods yielded identical results. Blank samples lacking either the protein or the metals were prepared in a similar manner. Samples were acid digested at 80°C for 1 h and then overnight at room temperature with concentrated HNO<sub>3</sub>. After digestion, H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 1.5%. Background metal levels in blank samples were < 15 % of the level detected in C-MBD samples.

Spectroscopic Analysis of Metal Binding to C-MBD-  $Zn^{+2}$  binding experiments using the  $Zn^{2+}$  binding chromophore mag-fura-2 were carried out as previously described (Liu et al., 2005; VanZile et al., 2000). 10  $\mu$ M C-MBD was titrated with 1 mM  $Zn^{+2}$  in the presence of 20  $\mu$ M mag-fura-2 (Molecular Probes, Eugene, OR) and the absorbance change at 366 nm was monitored. Free metal concentrations were calculated from  $K_I=[I.Zn^{+2}]/[I_{free}][Zn^{+2}]$ , where I is mag-fura-2 and  $K_I$  is the association constant of mag-fura-2 for  $Zn^{+2}$  (Liu et al., 2005). An extinction coefficient of 29900 M<sup>-1</sup> cm<sup>-1</sup> at 366 nm for metal-free mag-fura-2 and  $K_I$  of 5.0 x 10<sup>7</sup> M<sup>-1</sup> was used in determinations of free

mag-fura-2 and free  $Zn^{+2}$  (Walkup and Imperiali, 1997). The metal-protein association constant (K<sub>a</sub>) and the number of metal binding sites (n) in C-MBD, were calculated by fitting the data to  $v=nK_a[Metal]_f/(1+K_a[Metal]_f)$ , where v is the ratio of moles of metal bound to total protein (Guo and Giedroc, 1997). As above, reported errors for K<sub>a</sub> and n are asymptotic standard errors provided by the fitting software (Origin, OriginLab, Northampton, MA).

*Free Thiol Quantification*- A DTNB colorimetric assay was used to determine the number of reduced thiols in C-MBD (Bulaj et al., 1998). A standard calibration curve was prepared using L-Cysteine (Sigma). C-MBD (0.25 mg/ml) was added to a media containing 100  $\mu$ M Tris, pH 8.00, 2.5 mM sodium acetate, 1 mM ascorbic acid, 100  $\mu$ M DTNB. The reaction was allowed to go to completion for 30 min at 25°C. The molar concentration of thiolate anion was quantified at 412 nm ( $\epsilon$  = 13600 M<sup>-1</sup> cm<sup>-1</sup>).

*Circular Dichroism Spectroscopy*- C-MBD was passed through a Sephadex-G10 column (Sigma) equilibrated with 20 mM phosphate, pH 7.5, 100 mM NaF, and 1 mM TCEP, and adjusted to 5  $\mu$ M C-MBD in the presence and absence of 75  $\mu$ M metal (Zn<sup>+2</sup>, Co<sup>+2</sup>, Cu<sup>+</sup>). Circular dichroism data were recorded on an Aviv 60DS spectrometer with a 25 nm bandwidth, and were collected every 1 nm at 20°C. Background spectra recorded with buffer alone or buffer with metal were subtracted from the sample spectra. The data were analyzed in the Dichroweb site (www.cryst.bbk.ac.uk/cdweb) using the K2d analysis algorithm (Andrade et al., 1993; Lobley et al., 2002; Whitmore and Wallace, 2004).

*Carboxymethylation of Cysteines-* C-MBD (1 mg/ml) was incubated with 10 mM DTT in 100 mM Tris, pH 8.5, 150 mM NaCl buffer for 30 min at 25°C to reduce disulfide bonds. The reduced protein was incubated with 20 mM IAA for 30 min at 25°C in the dark. The

protein was washed in an Amicon Ultra-15 Centricon (Millipore) with 15 volumes 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM TCEP.

*Modification of Histidines with Diethyl Pyrocarbonate (DEPC)-* C-MBD (1 mg/ml) was incubated with 15 mM DEPC, 100 mM Tris, pH 7.0, and 150 mM NaCl buffer for 30 min at 25 °C. The modified protein was washed in an Amicon Ultra-15 Centricon (Millipore) with 15 volumes 20 mM HEPES, pH 7.0, 150 mM NaCl, and 1 mM TCEP. The number of DEPC modified His was determined spectrophotometrically ( $\epsilon$  = 3200  $M^{-1}$ cm<sup>-1</sup>) as previously described (Goto et al., 2000).

Sample Preparation for X-ray Absorption Spectroscopy (XAS) Analysis- 1 ml of 0.88 mM C-MBD was incubated with 0.88 mM  $Zn^{+2}$  or with 2.64 mM  $Zn^{+2}$  in the presence of 1 mM TCEP for 30 min at 4°C. The protein was washed in an Amicon Ultra-15 Centricon (Millipore) with 10 mM HEPES, pH 7.0. The stoichiometry of Zn:C-MBD was determined by AAS as described above.

*XAS Data Collection and Analysis* - Zn K-edge XAS data for C-MBD were collected on beam line X9B at the National Synchrotron Light Source at Brookhaven National Laboratory. The sample was placed in a polycarbonate sample holder and frozen in liquid nitrogen. Data were collected under ring conditions of 2.8 GeV and 120-300 mA using a sagitally focusing Si(111) double-crystal monochromator. The X-ray energy of the focused monochromatic beam was internally calibrated to the first inflection of a Zn foil spectrum (9660.7 eV). X-ray fluorescence data were collected using a 13-element Ge detector (Canberra). X-ray absorption near-edge structure (XANES) data were collected from ca. 9460-9860 eV and X-ray absorption fine structure (EXAFS) were collected from 9460-10640 eV. The primary vertical aperture was set to 0.3 mm for all samples. EXAFS data were analyzed using the program EXAFS123 (Padden et al., 2001) using parameters, including multiple-scattering parameters for His imidazole ligands, using FEFF8. Details of the data analysis are provided in the supplementary materials.

# **3.6 SUPPLEMENTARY INFORMATION**

Table S1. Addit	ional fits fo	or One Zn <sup>+2</sup>	bound CMBD Z	In K-edge
<b>EXAFS</b> Data				
$N_2S_2$ fit	2N	2S	2-imd	g.o.f.
r(abs-scat) (A):	1.97(1)	2.23(2)	1.97(1)	0.56
DW sig^2 (A^2):	0.003(1)	0.009(1)	0.003(1)	
E0 shift (eV):	2(2)	1(3)	2(2)	
N <sub>3</sub> S <sub>2</sub> fit	3N	2S	3-imd	g.o.f.
r(abs-scat) (A):	1.98(1)	2.27(2)	1.98(1)	0.50
DW sig^2 (A^2):	0.006(1)	0.012(2)	0.006(1)	
E0 shift (eV):	2(1)	8. (3)	2(1)	
N <sub>4</sub> S <sub>1</sub> fit	4N	1S	3-imd	g.o.f.
r(abs-scat) (A):	1.99(1)	2.32(3)	1.99(1)	0.48
DW sig^2 (A^2):	0.007(1)	0.008(2)	0.007(1)	
E0 shift (eV):	4(1)	16(4)	4(1)	
N <sub>4</sub> S <sub>2</sub> fit	4N	2S	3-imd	g.o.f.
r(abs-scat) (A):	2.00(1)	2.31(4)	2.00(1)	0.55
DW sig^2 (A^2):	0.008(1)	0.017(3)	0.008(1)	
E0 shift (eV):	2.91(2)	14(4)	2.91(2)	
$N_5S_1$ fit	5N	1S	3-imd	g.o.f.
r(abs-scat) (A):	2.00(1)	2.35(4)	2.00(1)	0.56
DW sig^2 (A^2):	0.009(1)	0.011(3)	0.009(1)	
E0 shift (eV):	4.53(1)	21(5)	4.53(1)	

EXAFS Data				
$N_2S_2$ fit	2N	2S	2-imd	g.o.f.
r(abs-scat) (A):	1.98(2)	2.16(4)	1.98(2)	0.80
DW sig^2 (A^2):	0.002(1)	0.011(3)	0.002(1)	
E0 shift (eV):	4(3)	-6(7)	4(3)	
$N_3S_2$ fit	3N	2S	3-imd	g.o.f.
r(abs-scat) (A):	1.99(1)	2.23(6)	1.99109	0.77
DW sig^2 (A^2):	0.003(1)	0.020(6)	0.00283797	
E0 shift (eV):	3(2)	6(6)	2.99093	
N <sub>4</sub> S <sub>1</sub> fit	4N	1S	3-imd	g.o.f.
r(abs-scat) (A):	2.00(1)	2.43(6)	2.00(1)	0.67
DW sig^2 (A^2):	0.004(1)	0.016(6)	0.004(1)	
E0 shift (eV):	5(1)	28(5)	5(1)	_
N <sub>4</sub> S <sub>2</sub> fit	4N	2S	3-imd	g.o.f.
r(abs-scat) (A):	2.00	2.55(13)	2.00	0.71
DW sig^2 (A^2):	0.004(1)	0.05(2)	0.004(1)	
E0 shift (eV):	6(1)	35(6)	6(1)	
$N_5S_1$ fit	5N	1S	3-imd	g.o.f.
r(abs-scat) (A):	2.00(1)	2.45(8)	2.00(1)	0.74
DW sig^2 (A^2):	0.006(1)	0.018(8)	0.006(1)	
E0 shift (eV):	5(1)	31(6)	5(1)	

Table S2.	Additional fi	ts for	Three	$Zn^{+2}$	bound	CMBD	Zn	K-edge
<b>EXAFS</b> Dat	ta							-

Table S3.	Additional fits for One Zn <sup>+2</sup> bound Cys-alkylated CMBD
Zn K-edge	EXAFS Data

N <sub>3</sub> fit	3N	2-imd		g.o.f.
r(abs-scat) (A):	1.99(2)	1.99(2)		0.82
DW sig^2 (A^2):	0.002(1)	0.002(1)		_
E0 shift (eV):	4(2)	4(2)		_
N <sub>5</sub> fit	5N	3-imd		g.o.f.
r(abs-scat) (A):	1.98(1)	1.98(1)		0.73
DW sig^2 (A^2):	0.004(1)	0.004(1)		_
E0 shift (eV):	3(1)	3(1)		_
$N_3S_1$ fit	3N	1S	3-imd	g.o.f.
r(abs-scat) (A):	1.94(3)	2.07(2)	1.94(3)	0.72
DW sig^2 (A^2):	0.006(2)	0.003(1)	0.006(2)	_
E0 shift (eV):	2(2)	-27(6)	2(2)	_

#### **XAS Data Analysis**

EXAFS data were analyzed using the program EXAFS123 (Padden et al., 2001). Five scans were averaged to generate an EXAFS spectrum. The summed data files were background corrected and normalized using a five section cubic spline to fit the background in the pre-edge and post-edge regions. The data were converted to *k* space using the relationship  $[2m_e(E - E_0)/\hbar^2]^{1/2}$  where  $m_e$  is the electron mass,  $\hbar$  is Plank's constant divided by  $2\pi$  and  $E_0$  is the threshold energy of the absorption edge, which was chosen to be 9670 eV. Least-squares fits of the EXAFS data over a *k* range of 2-12.5 Å<sup>-1</sup> was performed on unfiltered data. The upper limit of the *k* space range was limited by noise. Best fits were generated by minimizing the goodness of fit criterion  $(GOF = [n \{idp\}/(n \{idp\} - n \{p\})]^{1/2}R$ , where R = ave [data simulation/esd(data)], n(p) is the number of varied parameters, and n(idp) is the number of data points for unfiltered refinements or  $2(r_{max} - r_{min})(k_{max}-k_{min})\pi$  for filtered refinements), and minimizing the disorder value,  $\sigma^2$ , as derived by single-scattering EXAFS theory (equations 1 and 2).

Theoretical phases and amplitudes for EXAFS analyses were calculated using FEFF 8 and the crystallographically characterized model compounds:  $[Zn(S-2,3,5,6-Me_4C_6H)_2(1-Me-imd)_2]$ .<sup>2</sup> The phase and amplitude parameters from the calculations were then used to fit data obtained on the same model compounds used in the calculations in order to obtain values for  $\sigma^2_{model}$ .

$$Z_{c} = \sum_{shells} \{ NA[f(k)]k^{-1}r^{-2}e^{-2\sigma k^{2}} \sin(2kr + \alpha(k)) \}$$
(Padden et al., 2001)  
$$k = [4\pi m_{e}(E - (8340\text{eV}) + \Delta E))h]^{1/2}$$
(Toyoshima and Inesi, 2004)

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein

# Novel Zn<sup>2+</sup> Coordination by the Regulatory N-terminus Metal Binding Domain of *Arabidopsis thaliana* Zn<sup>2+</sup>-ATPase HMA2

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### **4.1 ABSTRACT**

Arabidopsis thaliana have three genes coding for  $Zn^{2+}$  transporting membrane ATPases. These play key roles maintaining metal homeostasis. They have unique sequences in both N- and C-termini that differentiate them from other P<sub>1B</sub>-type ATPases. We previously reported the metal binding characteristics and regulatory role of the Cterminal metal binding domain (C-MBD) present in Arabidopsis thaliana HMA2 (Eren and Argüello 2006). We now describe the properties of the single N-terminal metal binding domain (N-MBD) present in this Zn<sup>2+</sup>-ATPase. This 75 amino acid domain is homologous to Cu<sup>+</sup>-ATPases N-MBDs; however, the typical CXXC sequence is replaced by a Cys17CysThrSerGlu21 sequence. HMA2 N-MBD binds one Zn<sup>2+</sup>, Cd<sup>2+</sup> or Cu<sup>+</sup> ion per molecule. Mutagenesis studies indicate that Cys17, C18 and Glu21 participate in  $Zn^{2+}$ and Cd<sup>2+</sup> coordination while binding to HMA2 N-MBD. However, Cys17 and Glu21 (but not Cys18) are required for Cu<sup>+</sup> binding. These results suggest different coordination geometry for  $Zn^{2+}/Cd^{2+}$  and  $Cu^{+}$  ions binding to HMA2 N-MBD. Although the metal coordination is also distinct from that observed during metal binding to N-MBDs of Cu<sup>+</sup>-ATPases, homology modeling, and structure comparison by circular dichroism indicate that HMA2 C-MBD is likely to share the  $\beta\alpha\beta\beta\alpha$  fold present in Cu<sup>+</sup>-ATPase N-MBDs. ATPase activity measurement with the N-terminal truncated form of HMA2 showed that the N-MBD is required for maximum enzyme turnover rate without significantly affecting metal binding to transmembrane metal binding sites. HMA2 constructs carrying the mutations Cys17Ala, Cys18Ala and Glu21Ala/Cys showed kinetics similar to the truncated form. These results support the concept that metal binding to HMA2 N-MBD controls the enzyme turnover rate.

### **4.2 INTRODUCTION**

 $P_{1B}$ -type ATPases, a subfamily of P-type ATPases, transport transition metal ions (Ag<sup>+</sup>, Cu<sup>+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>, Co<sup>2+</sup>) across biological membranes using the energy of ATP hydrolysis (Axelsen and Palmgren, 1998; Axelsen and Palmgren, 2001; Argüello 2003; Williams and Mills, 2005). Found in almost all organisms, from archea to humans, these ATPases are key components of metal homeostasis mechanisms (Axelsen and Palmgren, 2001; Argüello 2003; Williams and Mills, 2003; Williams and Mills, 2003; Williams and Mills, 2005; Colangelo and Guerinot 2006). Some mutations in the coding sequence of the two human Cu<sup>+</sup>-ATPases cause Menkes and Wilson's diseases (Bull et al., 1993; Vulpe et al., 1993). Similarly, deletion of *Arabidopsis thaliana* P<sub>1B</sub>-ATPase gene expression leads to important imbalances in Zn<sup>2+</sup> and Cu<sup>+</sup> homeostasis (Eren and Argüello, 2004; Hussain et al., 2004; Hirayama et al., 1999).

Most  $P_{1B}$ -ATPases have 8 transmembrane helices (TM) (Axelsen and Palmgren, 1998; Axelsen and Palmgren 2001; Argüello, 2003; Melchers et al., 1996, Tsai et al., 2002). Conserved residues in TMs H6, H7 and H8 form the transmembrane metal binding domain (TMBD) and provide signature sequences that predict the corresponding metal specificity (Argüello, 2003; Mandal, 2004; Lowe, 2004; Dutta, 2006). The large cytoplasmic loop between TMs H6 and H7 is responsible for ATP binding and hydrolysis. This segment encompasses the nucleotide binding (N) and phosphorylation (P) domains (Sazinsky et al., 2006b). A smaller cytoplasmic loop located between H4 and H6 forms the actuator (A) domain (Sazinsky et al., 2006a, 2006b; Toyoshima and Inesi, 2004). In addition to TMBD, most  $P_{1B}$ -type ATPases have regulatory cytoplasmic metal

binding domains located in the N-terminus (N-MBD), C-terminus (C-MBD), or both (Arguello 2003, Eren et al., 2006, Lutsenko and Petris, 2003).

Typical N-MBDs present in Cu<sup>+</sup>-ATPases are usually 60-70 amino acids domains containing conserved CXXC signature sequences (Argüello, 2003; Arnesano et al., 2002; Lutsenko and Petris, 2003; Rensing et al., 1999). The high resolution structures of several of the Cu<sup>+</sup>-ATPases N-MBDs show a characteristic  $\beta\alpha\beta\beta\alpha\beta$  fold (Banci et al., 2001, 2002; Gitschier et al., 1998; Rosenzweig et al., 1999). These are homologous to well characterized Cu<sup>+</sup>-chaperones such as human Atox1, yeast Atx1 and bacterial CopZ (Arnesano et al., 2002; Banci et al., 2001, 2002; Gitschier et al., 1998; Rosenzweig et al., 1999). In vitro, N-MBDs bind various transition metals (Cu<sup>+</sup>, Ag<sup>+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>) (Banci et al., 2002; DiDonato et al., 1997; Gitschier et al., 1998; Harrison et al., 1999; Jensen et al., 1999; Lutsenko et al., 1997). In vivo, N-MBDs receive Cu<sup>+</sup> from the corresponding chaperones (Hamza et al., 1999; Huffman and O'Halloran, 2000; Larin et al., 1999; Strausak et al., 2003, Wernimont et al., 2000, 2004). Cu<sup>+</sup> binding to Cu<sup>+</sup>-ATPase N-MBDs appear to regulate enzyme activity. Truncation of the N-MBDs or removal of their metal binding capability by site-directed mutagenesis results in reduced enzyme activity with small or no changes in metal dependence for ATPase activation (Bal et al., 2001; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003; Mitra and Sharma, 2002; Voskoboinik et al., 1999, 2001). Studies using model Archaeglobulus fulgidus Cu<sup>+</sup>- and Cu<sup>2+</sup>-ATPase,s CopA and CopB, showed that N-MBDs control the enzyme turnover rate by affecting the rate limiting conformational change associated with metal release/dephosphorylation (Mana-Capelli et al., 2003; Mandal and Argüello, 2003). Lutsenko and co-workers have shown that Wilson disease protein N-MBDs interact with the ATP-BD in a Cu<sup>+</sup>-dependent manner (Tsivkovskii et al., 2001). Moreover, in the cases of Menkes and Wilson disease proteins, at least one N-MBD domain is required for the targeting of these proteins to the plasma membrane or a vesicular compartment, repectively (Forbes et al., 1999; Petris et al., 1996; Schaefer et al., 1999).

While the N-MBDs present in Cu<sup>+</sup>-ATPases are well characterized, distinct Nand C-MBDs present in eukaryotes (plants) Zn<sup>2+</sup>-ATPases received less attention. Characterization of the molecular function and *in planta* physiological roles of these domains is relevant since they are likely to regulate metal transport rates and protein targeting. Recently, we characterized the Arabidopsis thaliana Zn<sup>2+</sup>-ATPase HMA2 Cterminal metal binding domain (C-MBD) (Eren et al., 2006). Truncation of the C-MBD results in  $\approx 50\%$  decrease in HMA2 activity without significantly altering Zn<sup>2+</sup> or Cd<sup>2+</sup>  $K_{1/2}$  for ATPase activation. This suggests an auto stimulation by which cytoplasmic metal binding to C-MBD drives faster transport (Eren et al., 2006). HMA2 C-MBD binds three  $Zn^{2+}$  with high affinity, and  $Zn^{2+}$  is likely coordinated with 4 His in two sites and 3 His and 1 Cys in the other site, indicating a unique coordination of  $Zn^{2+}$  in plant P<sub>1B</sub>-type ATPases (Eren et al., 2006). All plant Zn<sup>2+</sup>-ATPases have relatively well conserved Ntermini, approximately 70 amino acids long, that appear similar to the typical N-MBDs observed in Cu<sup>+</sup>-ATPases. However, in these the key Cu<sup>+</sup> binding CXXC sequence is replaced with highly conserved CCXXE sequences (x = Ser, Thr, Pro, Ala) (Fig. 4.1A and B). It is possible that the two Cys and the acidic residue Glu could bind  $Zn^{2+}$  with a distinct coordination. Interestingly, although different from plant Zn<sup>+</sup>-ATPases, the N-MBDs present in the *E. coli*  $Zn^{2+}$ -ATPase ZntA containing a metal binding Asp58CysAlaAlaCys62 is similar to  $Cu^+$ -ATPase N-MBDs (Banci et al., 2002). However, the ZntA N-MBD binds and coordinates  $Zn^{2+}$  with the sulfur atoms of Cys59 and Cys62 and the oxygen of Asp58 (Banci et al., 2002).

Here, we describe the characterization of the N-MBD present in the Arabidopsis  $Zn^{2+}$ -ATPase. This N-MBD contains the Cys17CysThrSerGlu21 sequence. The ATPase kinetics of N-terminal truncated HMA2 ( $\Delta$ N-HMA2) (lacking the first 75 amino acids), and Cys17Ala, Cys18Ala, Ser20Ala, Ser20Cys, Glu21Ala and Glu21Cys mutants mutants was characterized. In addition the metal binding properties of isolated N-MBDs (wild type (wt) and Cys17Ala, Cys18Ala, Ser20Ala, Ser20Ala, Ser20Cys, Glu21Ala and Glu21Cys mutants) have been determined. Results from these experiments together with homology modeling, suggest that HMA2 N-MBD controls enzyme turnover by binding Zn<sup>2+</sup> with a distinct coordination.

### RESULTS

Zn<sup>2+</sup>-ATPases have been identified in a number of plants including *A. thaliana, A. halleri, Oryza sativa, Thlaspi caerulescens, Zea mays* and *Sorghum bicolor* (Williams and Mills, 2005). Sequence alignment of plant Zn<sup>2+</sup>-ATPase N-termini showed that these share significant homology (25-30%) with Cu<sup>+</sup>-ATPases N-MBDs. However, all plant Zn<sup>2+</sup>-ATPase N-termini lack the characteristic CXXC conserved sequences of Cu<sup>+</sup>-ATPase N-MBDs (Fig. 4.1A).

Α

AfCopA		MVKDTYISSASKTPPMERTVRVTGMT C AMC VKSIETAVGSLEGVEEVRVNLATETAFIRF	60
BsCopZ		$MEQKTLQVEGMS \mathbf{C} QH \mathbf{C} VKAVETSVGELDGVSAVHVNLEAGKVDVS F$	46
HsATP7A	4-NMBD	SLQKIPLNVVSQPLTQETVINIDGMTCNSCVQSIEGVISKKPGVKSIRVSLANSNGTVEY	421
HsATP7B	4-NMBD	HSPGSPPRNQVQGTCSTTLIAIAGMTCASCVHSIEGMISQLEGVQQISVSLAEGTATVLY	403

#### В

Mt Cd <sup>2+</sup> -ATPase	MSSSEGGKKLQKSYFDVVGLCCSSEVPLIENILKPLQGVKEVSVIVPSRT	50
TcHMA4	MALQKEIKNKEEDKKTKKKWQKSYFDVLGI <b>CC</b> TS <b>E</b> IPLIENILKSLDGVKEYTVIVPSRT	60
AhHMA4	MASQNKEEEKKKVKKLQKSYFDVLGI <b>CC</b> TS <b>E</b> VPIIENILKSLDGVKEYSVIVPSRT	56
AtHMA4	MALQNKEEEKKKVKKLQKSYFDVLGICCTSEVPIIENILKSLDGVKEYSVIVPSRT	56
AtHMA2	MASKKMTKSYFDVLGICCTSEVPLIENILNSMDGVKEFSVIVPSRT	46
AhHMA3	MAEGEEAKKKNLQTSYFDVVGICCTSEVSIVGDVLRPLDGVKEFSVIVPSRT	52
AtHMA3	MAEGEESKKMNLQTSYFDVVGICCSSEVSIVGNVLRQVDGVKEFSVIVPSRT	52
MtCof	MVENIKRSNFEVLGMCCATEATLVERILKPLHGVKAVSVIVPART	45
OsHMA2	AAEEPTRCGGGGGGGGGGRKRKKTYLDVLGVCCSAEVALVERLLAPLDGVRVVSVVVASRT	81
OsHMA3	MAAEGGRCQKSYFDVLGI <b>CC</b> PS <b>E</b> VPLVEKLLQPLEGVQKVTVIVPSRT	48
OtQ01EZ3	TAATIAAKLALEARGGNEPSTRTRLSVNG I CC PSE VPLIHKILGKLEGVREVKVIVPTKT	63

Figure 4.1 Multiple alignment of typical Cu<sup>+</sup>-ATPases N-MBDs and Cu chaperones (A) and plant Zn<sup>2+</sup>-ATPases N-MBDs (B). Positions of the last amino acid are indicated. Conserved amino acids in the CXXC or the CCXXE regions are indicated in bold. Accession numbers for the different proteins are: *Archeoglobus fulgidus* CopA, O297777; *Bacillus subtilis* CopZ, O32221; *Homo sapiens* ATP7A, Q04656; *H. sapiens* ATP7B, P35670; *Thlaspi caerulescens* HMA4, Q70LF4; *Arabidopsis halleri* HMA3, Q70Q04; *A. halleri* HMA4, Q3ZDL9; *A. thaliana* HMA2, Q9SZW4; *A. thaliana* HMA3, Q9SZW5; *A. thaliana* HMA4, O64474; *Medicago*  *truncatula* Cof, Q1SYW8; *M. truncatula* Cd<sup>2+</sup>-ATPase Q1T1H3; *Oryza sativa* HMA2, AP004278; *O. sativa* HMA3, Q8H384; *Ostreococcus tauri* Q01EZ3.

Instead all known, plant  $Zn^{2+}$ -ATPases contain highly conserved CCXXE (X= S,T,P or A) sequences in their N-termini (Fig. 4.1B). Both Cys and Glu side chains can participate in  $Zn^{2+}$  coordination (Banci et al., 2002). In order to determine whether plant  $Zn^{2+}$ -ATPases N-termini function as regulatory domains and contain novel metal binding sites, we characterized *A. thaliana* HMA2 Zn<sup>2+</sup>-ATPase N-MBD.

#### 4.3.1 Homology Modeling of N-MBD

The conserved CXXC domain of Cu<sup>+</sup>-ATPases localizes at the end of the first loop and the first turn of the first  $\alpha$  helix of the N-MBD (Gitschier et al., 1998; Banci et al., 2001), a region which is solvent accessible, and therefore, ready to interact with other proteins and receive Cu<sup>+</sup> via ligand exchange.Homology modelling was used to assess whether the Cys17CysThrSerGlu21 motif of the HMA2 N-MBD localizes in a similar region. *Helicobacter pylori* CopZ (Accession number: 1yg0) was identified, after running a Fasta search in the Protein Data Bank, as the structure which shared the highest degree of identity (32%) with HMA2 N-MBD. *H. pylori* CopZ and modelled N-MBD present the same  $\beta\alpha\beta\beta\alpha$  fold (Fig. 4.2A). In addition, the conserved CXXC and CCXXE motifs (Cys12 and 15 in *H. pylori* CopZ, and Cys17, Cys18 and Glu21 in N-MBD) overlap when both structures are compared. Interestingly, if Glu21 is changed to a Cys, the predicted topology of the N-MBD recreates the Cu<sup>+</sup>-binding site of CopZ (Fig 4.2B). When this region is modelled after *Escherichia coli* ZnTA Zn<sup>2+</sup>-P<sub>1B</sub>-ATPase N-MBD, the side chains involved in Zn<sup>2+</sup> coordination overlap (Cys17, Cys18 and Glu21 of N-MBD overlap Asp58, Cys59 and Cys62, respectively, data not shown). Circular dichroism (CD) analysis of the isolated HMA2 N-MBD was performed in order to validate the model. The analysis of the CD data revealed that N-MBD had the same proportion of secondary structure elements as N-MBDs of typical Cu<sup>+</sup>- ATPases, such as *A. fulgidus* CopA (Fig. 4.2C), which suggests a similar tertiary structure.



Figure 4.2 Structural aspects of HMA2 N-MBD. A. Homology modelling of HMA2 N-MBD (right) using the structure of *Helicobacter pylori* CopZ (1yg0) as

template (left). Amino acids relevant to metal binding are indicated in both structures. *B*. Superposition of the metal binding region of 1yg0 and the E21C mutated N-MBD. The coincidence of the cysteine residues is indicated. *C*. Circular dichroism analysis of N-MBD (–) and N-MBD of *Archeoglobus fulgidus* CopA (…). Secondary structure elements are indicated in the table. *a.h.*,  $\alpha$ -helix, *b.s.*,  $\beta$ -sheet, and *r.c.*, random coil. The values are given in percentages.

#### **4.3.2 Metal Binding to N-MBD**

In order to determine if the conserved residues in the CysCysThrSerGlu sequence contributes to metal coordination, as implied by sequence alignment and modelling, isolated soluble N-MBD (amino acids 1 to 75) carrying individual mutations Cys17Ala, Cys18Ala, Ser20Ala, Ser20Cys, Glu21Ala and Glu21Cys were obtained. All these mutants and wild type (wt) N-MBD were purified by affinity chromatography ( $\approx$ 90% purity, data not shown). CD analysis showed that all the mutant proteins have a similar folding to that of the wt N-MBD, indicating that these mutations do not have a profound effect on N-MBD secondary structure (data not shown). Table 4.1 shows the determination of metal binding to isolated wt N-MBD and N-MBD mutants by Atomic Absorption Spectroscopy (AAS). Our results indicate that under saturating conditions, wt N-MBD binds  $Zn^{2+}$  and  $Cd^{2+}$  with a stoichiometry of one metal/N-MBD. Interestingly, N-MBD also binds Cu<sup>+</sup> but not Cu<sup>2+</sup> and Co<sup>2+</sup>. N-MBD carrying mutations Cys17Ala, Cys18Ala or Glu21Ala does not bind  $Zn^{2+}$  or  $Cd^{2+}$ . In addition, while the Cys17Ala and Glu21Ala mutants are unable to bind Cu<sup>+</sup>, the Cys18Ala mutation does not affect Cu<sup>+</sup> binding. Replacement of Glu21 with another metal coordinating residue, Cys, also inhibited  $Zn^{2+}$  and  $Cd^{2+}$  binding, indicating that the presence of an acidic residue is

critical for the coordination of these metals. However, the Glu21Cvs mutant is still able to bind Cu<sup>+</sup>. N-MBD carrying mutations Ser20Ala orSer29Cys served as control of inespecific local structure perturbation. These results suggest that Zn<sup>2+</sup> and Cd<sup>2+</sup> would likely have a tetrahedrical coordination involving Cys17, Cys18 and Glu21, and a water molecule. Alternatively,  $Cu^+$  appears to be coordinated by Cys17 and Glu21.

	Metal bound / N-MBD				
	Zn <sup>2+</sup>	$Cd^{2+}$	Co <sup>2+</sup>	Cu <sup>+</sup>	Cu <sup>2+</sup>
N-MBD	$0.99\pm0.10*$	$1.20\pm0.21$	$0.01\pm0.00$	$1.03\pm0.15$	$0.14\pm0.01$
C17A	n.d.**	$0.15\pm0.03$	$0.1\pm0.04$	$0.16\pm0.07$	$0.15\pm0.08$
C18A	$0.21\pm0.05$	$0.16\pm0.05$	n.d.	$0.93\pm0.15$	$0.12\pm0.05$
S20A	$1.05\pm0.07$	$1.20\pm0.10$	$0.23\pm0.05$	$1.04\pm0.20$	$0.11\pm0.01$
S20C	$0.83\pm0.15$	$1.08\pm0.13$	n.d.	$1.04\pm0.23$	$0.04\pm0.00$
E21A	n.d.	$0.09\pm0.02$	$0.17 \pm 0.06$	$0.25 \pm 0.06$	n.d.
E21C	$0.2 \pm 0.01$	n.d.	$0.20 \pm 0.03$	$1.00 \pm 0.32$	$0.23 \pm 0.20$
*Values are th	$a maan \pm SE(r)$	1-3)			

Table 4.1 Determination of metal binding stoichiometry to N-MBD by AAS.

Values are the mean  $\pm$  S.E. (*n*=3).

\*\*n.d. not detected (under background levels).

 $Zn^2$  and  $Cd^{2+}$  Titrations of N-MBD. Although the N-MBD is able to bind different cations, the affinity for each one of them might differ. To investigate this aspect and the effect of the mutations on metal selectivity, we determined the affinity of N-MBD for  $Zn^{2+}$  and  $Cd^{2+}$ . Wt N-MBD and N-MBD mutants were titrated with  $Zn^{2+}$  and  $Cd^{2+}$  in the presence of the fluorescence indicator, mag-fura-2. Mag-fura-2 forms a 1:1 complex with the indicated metals and its K<sub>a</sub> for these metals was previously calculated (Liu et al., 2005). No binding of  $Zn^{2+}$  and  $Cd^{2+}$  could be detected in the Cys17Ala, Cys18Ala, Glu21Ala and Glu21Cys mutants (Table 4.2), in agreement with our AAS data (Table 4.1). Wt-NMBD had no significant preference for  $Zn^{2+}$  or  $Cd^{2+}$  (Fig. 4.3A and B, Table 4.2). Moreover, the  $K_a$  for  $Zn^{2+}$  or  $Cd^{2+}$  binding to Ser20Ala or Se20Cys mutants did not significantly differ from those of wt N-MBD, suggesting that these mutations do not affect metal binding (Table 4.2).



**Figure 4.3 Zn<sup>2+</sup> binding to N-MBD.** *A*. Representative spectra of titration of 10  $\mu$ M N-MBD and 20  $\mu$ M mag-fura-2 with increasing Zn<sup>2+</sup> concentrations. The arrow shows the direction of absorbance change at 366 nm as increasing concentrations of Zn<sup>2+</sup> are added. *B*. Determination of K<sub>a</sub> for Zn<sup>2+</sup> binding, and the number of metal binding sites in N-MBD. The data were fit to  $\nu = nK_a[Zn^{2+}]_{f'}(1+K_a[Zn^{2+}]_{f})$  with  $n = 0.97 \pm 0.08$  and  $K_a = 5.59 \pm 2.19 \times 10^6 \text{ M}^{-}$ . Values are the mean  $\pm$  S.E. (n = 3).

	Zn <sup>2+</sup>		Cd <sup>2</sup>	$Cd^{2+}$		
	Stoichimetry	Ka (10 <sup>6</sup> M)**	Stoichimetry	Ka (10 <sup>6</sup> M)		
N-MBD	$0.97\pm0.08*$	$5.59\pm2.19$	$1.00 \pm 0.12$	$3.74 \pm 1.37$		
C17A	n.d.	n.d.	n.d.	n.d.		
C18A	n.d.	n.d.	n.d.	n.d.		
S20A	$0.93\pm0.14$	$5.02 \pm 1.93$	$0.72 \pm 0.18$	$5.47\pm3.52$		
S20C	0.75	$5.04\pm2.75$	$1.02 \pm 0.10$	$8.66 \pm 2.32$		
E21A	n.d.	n.d.	n.d.	n.d.		
E21C	n.d.	n.d.	n.d.	n.d.		

Table 4.2 Zn<sup>2+</sup> and Cd<sup>2+</sup> binding stoichiometry and Ka of HMA2 N-MBD.

\*Values are the mean  $\pm$  S.E (*n*=3)

\*\* Parameters are those obtained by fitting the titration of 10 mM N-MBD and 20 mM Mag-Fura-2 with increasing  $Zn^{2+}$  or  $Cd^{2+}$  as shown in Fig. 4.3.

# 4.3.3 Effect of N-MBD Truncation and Mutation of Conserved Residues on HMA2 ATPase Activity

 $Zn^{2+}$ -dependent ATPase activity of HMA2, HMA2 lacking the N-MBD ( $\Delta$ N-HMA2) and mutant HMA2 were studied to assess the N-MBD functional role. Truncation of the N-MBD resulted in a 56% decrease in V<sub>max</sub> without significant changes in metal affinity (Fig. 4.4A and Table 4.3). A similar decrease of V<sub>max</sub> in the ATPase activities (36-60 %) was also observed in the HMA2 constructs carrying the individual mutations Cys17Ala, Cys18Ala, Glu21Ala and Glu21Cys. On the other hand, both Ser20Ala and Ser20Cys mutants showed a V<sub>max</sub> similar to that of wt HMA2 (Fig. 4.4A and Table 4.3). We did not observe a significant change in metal affinity with any of the HMA2 mutants (Table 4.3) indicating that N-MBD affects enzyme velocity without affecting metal binding to the TMBS. In these ATPase activity determinations the variations in levels of expression of the different HMA2 constructs, less than 15%, was taken into consideration (Fig. 4.4B).



Figure 4.4 Expression and ATPase activity of HMA2,  $\Delta$ N-HMA2 and N-MBD mutated HMA2. *A*. Zn<sup>+2</sup> dependent ATPase activity of HMA2 constructs. Values are the mean  $\pm$  S.E (*n*=3) normalized to its relative expression levels of each protein. Fitting parameters are listed in Table 3. *B*. Expression levels of HMA2 ( $\bigcirc$ ),  $\Delta$ N-HMA2 ( $\oplus$ ), and C17A ( $\Box$ ), C18A ( $\blacksquare$ ), S20A ( $\diamondsuit$ ), S20C ( $\blacklozenge$ ), E21A ( $\triangle$ ) and E21C ( $\blacktriangle$ ) mutants. Dot immunoblots were prepared at two different concentrations.

HMA2 Constructs	Κ <sub>1/2</sub> (μΜ)	V <sub>max</sub> (µmol/mg/h)
HMA2	$0.10\pm0.02$	$5.18\pm0.27$
ΔΝ-ΗΜΑ2	$0.09\pm0.02$	$2.28\pm0.12$
C17A	$0.09\pm0.02$	$3.30\pm0.14$
C18 A	$0.12\pm0.02$	$4.21\pm0.13$
S20A	$0.09\pm0.03$	$4.94\pm0.30$
S20C	$0.08\pm0.02$	$5.19\pm0.24$
E21A	$0.11 \pm 0.02$	$2.93 \pm 0.11$
E21C	$0.08\pm0.03$	$2.08 \pm 0.17$

Table 4.3 Kinetic parameters of HMA2  $\Delta$ N-HMA2 and N-MBD mutated HMA2.

\* Values are the mean  $\pm$  S.E (*n*=3).

### **4.4 DISCUSSION**

P<sub>1B</sub>-type ATPases contain cytoplasmic regulatory metal binding domains in their N-termini or C-termini or both. Most Cu<sup>+</sup>-ATPases and some bacterial Zn<sup>2+</sup>-ATPases have 60 to 70 amino acid long N-MBDs that are characterized by CXXC signature sequences. The involvement of both Cys in metal coordination has been shown (DiDonato et al., 1997; Lutsenko et al., 1997; Gitschier et al., 1998; Harrison et al., 1999; Jensen et al., 1999; Banci et al., 2002). Compared to other eukaryotes, plants have an unusually high number of P<sub>1B</sub>-ATPases (eight or nine) with distinct metal selectivities. Multiple sequence alignment studies show that although plant Cu<sup>+</sup>-ATPases contain the CXXC signature sequences in their N-termini, all plant Zn<sup>2+</sup>-ATPases lack this conserved sequence. Instead, all plant  $Zn^{2+}$ -ATPases contain a conserved CCXXE (X=Ser, Thr, Pro or Ala) sequence in their N-termini. However, neither the metal binding to these domains, nor their putative functional role has been previously established. Towards understanding the function of plant  $Zn^{2+}$ -ATPase N-termini we characterized the HMA2 N-terminus by investigating its metal binding capabilities, the involvement of conserved residues in metal coordination and its role in the enzyme ATPase activity.

#### 4.4.1 Metal Binding Capability of N-MBD

Both AAS analysis and metal titration studies showed that HMA2 N-MBD can bind  $Zn^{2+}$  and  $Cd^{2+}$  with a stoichiometry of one metal/N-MBD with high affinity. Our results indicated that both  $Zn^{2+}$  and  $Cd^{2+}$  are likely coordinated by two Cys (Cys17 and Cys18) and the Glu21 of the conserved CCXXE. A similar coordination has been observed for bacterial  $Zn^{2+}$ -ATPase ZntA N-MBD where the  $Zn^{2+}$  is coordinated by the thiol groups of Cys (Cys59 and Cys62) of the conserved CXXC domain and a carboxyl

group of an Asp residue (Asp58) (Banci et al., 2002). It has been proposed that the  $Zn^{2+}$ coordination is tetrahedral and the fourth ligand is possibly the oxygen of a H<sub>2</sub>O molecule. This idea is supported by the remarkably high solvent accessibility of this site (Banci et al., 2002). Our results showed that under metal saturating conditions HMA2 N-MBD can also bind Cu<sup>+</sup> with a stoichiometry of one. This is not surprising since ZntA N-MBD has also been shown to bind a variety of non-substrate metal ions including Cu<sup>+</sup> and Ag<sup>+</sup> (Rensing et al., 1998a; Sharma et al., 2000). Similarly, it has been shown that the Wilson disease protein N-MBD can also bind a number of metals including  $Cd^{2+}$ ,  $Zn^{2+}$ , Ni<sup>2+</sup> and Co<sup>2+</sup> (DiDonato et al., 1997; Lutsenko et al., 1997; DiDonato et al., 2002). The affinity for Cu<sup>+</sup>, however, was higher than to any of these metals. It has been proposed that Zn<sup>2+</sup> coordination in Wilson disease protein is different from that of Cu<sup>+</sup>, and His are also involved in coordination of  $Zn^{2+}$  (DiDonato et al., 2002). Our metal binding studies of HMA2 N-MBD mutants showed that the mutations Cys18Ala and Glu21Cys do not have any effect on Cu<sup>+</sup> binding, while replacements Cys17Ala and Glu21Ala abolish Cu<sup>+</sup> binding. The Glu21Cys mutation does not result in a loss of Cu<sup>+</sup> binding, most probably because it recreates the CXXC binding site, as shown in Fig 2B. This would imply that Cu<sup>+</sup> and Zn<sup>2+</sup> are differentially coordinated, Cys17, Cys18 and Glu21 are likely involved in  $Zn^{2+}$  binding, whereas only Cys17 and Glu21 coordinate  $Cu^{+}$ . This is in agreement with previous work where  $Cu^+$  has been shown to be coordinated linearly in the CXXC domain (Gitschier et al., 1998; Ralle et al., 2003), whereas Zn<sup>2+</sup> is tetrahedrically coordinated by two sulphydryl, a carboxyl and an external group (Banci et al., 2002; DiDonato et al., 2002). Although HMA2 N-MBD coordinates non-substrate Cu<sup>+</sup>, the presence of the Glu residue probably increases the affinity of this metal binding site for  $Zn^{2+}$  since the presence of the negative charges will provide a more favourable electrostatic contribution for more positively charged  $Zn^{2+}$  over  $Cu^+$  (Banci et al., 2002). Also Glu might also have a role in the context of hard/soft acid base theory; the Glu oxygen atom will stabilize binding of the harder, less polarizable  $Zn^{2+}$ , in contrast to the highly polarizable ion  $Cu^+$ .

#### **4.4.2 Functional Role of N-MBD**

To determine how the N-MBD affects the enzyme activity, we measured the metal dependent ATPase activity of the  $\Delta N$ -truncated HMA2 and compared it to that of the intact HMA2. Our results showed that truncation of the N-MBD resulted in a  $\approx 50\%$ decrease in V<sub>max</sub> without significantly altering metal affinity. Therefore, although N-MBD is required for maximum enzyme turnover rate, it does not influence the metal binding to TMBDs. Similar results have been observed with Cu<sup>+</sup>-ATPases (Mandal et al., 2003), Cu<sup>2+</sup>-ATPases (Mana-Capelli et al., 2003) and bacterial Zn<sup>2+</sup>-ATPases (Mitra and Sharma, 2001). ATPase activity measurements with the HMA2 mutants indicate that a mutation in the metal coordinating residues Cys17Ala, Cys18Ala and Glu21Ala results in a decrease in enzyme  $V_{max}$  in a similar manner with the wt HMA2. This result indicates that the decrease in the enzyme activity is dependent on metal binding ability of this domain and simply is not due to misfolding of the protein due to truncation since we have shown that these mutations do not affect N-MBD folding. This result is further supported by high activities observed with metal binding Ser20 mutations. HMA2 N-MBD apparently regulates enzyme turnover through affecting the rate limiting step of the catalytic activity. We have observed that HMA2 C-MBD also regulates the enzyme

turnover rate (Eren et al., 2006). Interestingly, truncation of both the N-MBD and the C-MBD results in a similar decrease in  $V_{max}$  when either of the domains is truncated, indicating that these domains might function in a coordinated manner (Eren et al. 2006). It was shown that the Wilson disease protein N-MBD interacts with the ATP-BD (Tsivkovskii et al., 2001). It is possible that N-MBD regulates enzyme activity through interacting with cytoplasmic domains that undergo key conformational changes during the catalytic cycle (Tsivkovskii et al., 2001). However, further studies are required to investigate if plant N-MBD domains interact with any of the other cytoplasmic domains (A-, P-, or N- or C-MBD).

In summary, HMA2 N-MBD appears as a regulatory domain that is required for maximum enzyme turnover rate. Our results indicate that HMA2 N-MBD, as well as those in other plant  $Zn^{2+}$ -ATPases, have novel metal binding sites with  $Zn^{2+}$  coordinated by the two Cys and the Glu residue of the conserved CysCysXXGlu sequence probably in a tetrahedral geometry.

### **4.5 MATERIALS AND METHODS**

Cloning and Expression of HMA2 Constructs- The yeast expression vector pYES2/CT carrying A. thaliana HMA2 containing a C-terminal Strep-tag (WSHPQFEK) was prepared as previously described. N-terminus truncated HMA2 ( $\Delta$ N-HMA2) lacking the N-terminal first 75 amino acids, starting from Val76 and ending in Glu951, with a Cterminal Strep-tag was amplified from a previously prepared HMA2-pPRIBA1/Strep the oligonucleotides: 5'construct by using GCGGTACCAAAAATGGTAACCGGAGAACCAA and 3'-GCGTTTAAACTTATTTTCGAACTGC{Eren, 2006 #201}. The amplicon was ligated into the KpnI and PmeI sites of the yeast expression vector pYES2/CT (Invitrogen, Carlsbad, CA).

C17A, C18A, S20A, S20C, E21A and E21C HMA2 mutants were amplified 5' oligonucleotides: 5'using the C17A. CAACGGTACCTCCGATGTACAAGCAATTCC; 5'-C18A, ATTTGCGCTACATCGGAGGTACCGTTGAT; S20A, 5'-ATTTGCTGTACAGCGGAGGTACCGTTGAT; 5'-S20C, ATTTGCTGTACATGCGAGGTACCGTTGAT; E21A, 5'-ATTTGCTGTACATCGGCGGTACCGTTGAT; E21C. 5'-

ATTTGCTGTACTTCGTGTGTGTACCGTTGAT and the complementary 3' primers using HMA2-pYES2/Strep as a template. The resulting linear mutant HMA2-pYES2/Strep amplicons were transformed into *E. coli* TOP10 cells (Invitrogen).

Isolated wild type and mutant HMA2 coding plasmids were then transformed into yeast strain INVSc1 *MAT* $\alpha$ *his3* $\Delta$ *1 leu2 trp1-289 ura3-52* (Invitrogen) by electroporation

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of cells at 1.5 kV, 25  $\mu$ F, 200 $\Omega$ . Sequence was verified by automated DNA sequencing (MacrogenUSA, Rockville, MD). Expression of HMA2 constructs in yeast was performed as previously described.

*Cloning, Expression and Purification of HMA2 N-MBDs-* A cDNA coding for the first 75 amino acids of HMA2, from Met1 to Arg75 was amplified by using the oligonucleotides: 5'-GCAGGTACCGCGTCGAAGAAGATG and 3'-TCGCTCGAGCCTCACATTTGCTTCTAACTG and HMA2 cDNA as a template.

Resulting amplicon was cloned into the *KpnI* and *XhoI* sites of the bacterial expression vector pPRIBA1 (N-MBD-pPRIBA1). This introduces a Strep-tag into the C-terminal end of the protein. *E. coli* BL21(DE3)pLysS cells were transformed with this vector. C17A, C18A, S20A, S20C, E21A and E21C N-MBD mutants were amplified using the oligonucleotides listed above and N-MBD-pPRIBA1 as a template. The resulting linear N-MBD-pPRIBA1 amplicons were transferred into *E. coli* BL21Star(DE3)pLysS (Invitrogen) cells.

N-MBD expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cells were collected by centrifugation at 2500 x g for 5 min and resuspended in 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Purification of N-MBDs was performed using Strep-tag affinity chromatography, as previously described for the C-MBD of HMA2 (Eren et al., 2006). Purified protein was concentrated to 2 mg mL<sup>-1</sup> using an Amicon Ultra-15 Centricon (Millipore, Billerica, MA) and stored in 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM TCEP at -80°C. Routine protein concentration determinations were performed in accordance to Bradford (1976) using bovine serum albumin as a standard.
*Yeast Membrane Preparation*- Membranes from yeast were prepared as previously described (Voskoboinik et al., 2001; Eren and Argüello, 2004). SDS-PAGE was carried out in 10% acrylamide gels (Laemmli, 1970). Heterologous proteins in the membrane preparations were detected by electroblotting the gels onto nitrocellulose membranes and immunostaining with Strep-Tactin horseradish peroxidase antibody (IBA). The relative expression levels of each protein was evaluated as previously described (Mana-Capelli et al., 2003; Eren et al., 2006). Briefly, equal amounts of each membrane preparation were subjected to a 1:2 serial dilution. These were blotted onto a nitrocellulose membrane, immunostained, and integrated density values were quantified using AlphaImager software (Alpha Innotech Corp., San Laendro, CA).

*ATPase Assays-*  $Zn^{2+}$  dependent ATPase activity determinations were performed as previously described in a medium containing 50 mM Tris, pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM ATP, 20 mM cysteine, 1 mM dithiothreithrol (DTT), 0.5 mg mL<sup>-1</sup> saponin, and 40 µg mL<sup>-1</sup> protein (membrane preparation), at 30°C (Eren and Argüello, 2004). The concentrations of ZnCl<sub>2</sub> were varied as indicated in the figures. ATPase activity measured in the absence of metal was always < 10 % of V<sub>max</sub> and was subtracted from plotted values.

Characterization of Metal Binding Stoichiometry and Metal Affinity of wild type and *mutant N-MBDs-* Total metal binding capacity was measured by atomic absorption spectroscopy (AAS) (AAnalyst 300, Perkin-Elmer, Foster City, CA), as previously described (Eren and Argüello, 2004). Briefly, 100 µM wild type (wt) or mutant N-MBDs in 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM TCEP were incubated with 1.0 mM of metal at 4°C for 30 min. For Cu (II) binding determinations TCEP was removed by

passage through a Sephadex G-10 column prior to metal binding. Excess metal was removed by passage through a Sephadex G-10 column (Sigma, St Louis, MO) and samples were acid digested at 80°C for 1 h and then overnight at room temperature with concentrated HNO<sub>3</sub>. After digestion,  $H_2O_2$  was added to a final concentration of 1.5%. Background metal levels in blank samples were < 10 % of the level detected in N-MBD samples.

 $Zn^{+2}$  binding titrations were carried out to determine affinity (Liu et al., 2005). 10  $\mu$ M protein was titrated with 1 mM  $Zn^{+2}$  or  $Cd^{2+}$  in the presence of 20  $\mu$ M mag-fura-2 (Molecular Probes, Eugene, OR) in 10 mM BisTris , pH 7.0, 1 mM TCEP buffer and the absorbance change at 366 nm was monitored.

*Circular Dichroism Spectroscopy*- Wild type and mutant N-MBDS were passed through a Sephadex-G10 column (Sigma) equilibrated with 20 mM phosphate, pH 7.5, 100 mM NaF, and 1 mM TCEP. Proteins were diluted to 10  $\mu$ M in in the absence or presence of 100  $\mu$ M Zn<sup>2+</sup> and placed in a 1 mm quartz cuvette. Circular dichroism data were recorded on an Aviv 60DS spectrometer with a 25 nm bandwidth, and data were collected every 1 nm at 25°C. Background spectra were recorded with buffer alone or buffer with metal and were subtracted from all recorded spectra. The data were analyzed using the Dichroweb site (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) with the K2d analysis algorithm (Andrade et al., 1993; Lobley et al., 2002; Whitmore and Wallace, 2004).

*Multiple Alignment and Homology Modelling of HMA2 N-MBD-* Sequence comparison of N-MBDs from typical Cu<sup>+</sup>-ATPases and plant Zn<sup>2+</sup>-ATPases was perfomed by means of CLUSTALW (Thompson et al, 1994). A suitable template for homology modelling of

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HMA2 N-MBD, *Helicobacter pylori* CopZ 1yg0, was identified by performing a Fasta search (Pearson, 1990) in the Protein Data Bank repository of protein structures. HMA2 N-MBD structures were modelled using SWISS-MODEL (Guex and Peitsch, 1997; Peitsch, 1995, Schwede et al., 2003) and were visualized with PyMol (Delano Scientific, Palo Alto, CA).

*Data Analysis*- Curves of ATPase activity vs. metal concentration were fit to  $v=V_{max}L/(L+K_{1/2})$ , where L is the concentration of variable ligand. The reported standard errors for  $V_{max}$  and  $K_{1/2}$  are asymptotic standard errors reported by the fitting software (Kaleidagraph, Synergy, Reading, PA). In metal binding experiments, free metal concentrations were calculated from  $K_I=[I.Zn^{+2}]/[I_{free}][Zn^{+2}]$ , where I is mag-fura-2 and  $K_I$  is the association constant of mag-fura-2 for  $Zn^{+2}$  (Liu et al., 2005). An extinction coefficient of 29,900 M<sup>-1</sup> cm<sup>-1</sup> at 366 nm for metal-free mag-fura-2 and  $K_I$  of 5.0 x 10<sup>7</sup> M<sup>-1</sup> was used in determinations of free mag-fura-2 and free  $Zn^{+2}$  (Liu et al., 2005). The metal-protein  $K_a$  and the number of metal binding sites (n) in N-MBD, were calculated from  $v=nK_a[Metal]_{t'}(1+K_a[Metal]_t)$ , where v is the ratio of moles of metal bound to total protein (Guo and Giedroc, 1997). As above, reported errors for  $K_a$  and n are asymptotic standard errors provided by the fitting software (Origin, OriginLab, Northampton, MA). In all cases, points are the mean  $\pm$  SE of at least three experiments performed with independent protein preparations.

I may not have gone where I intended to go, but I think I have ended up where I needed to be.

Douglas Adams

## **5. CONCLUSION**

 $Zn^{2+}$  is an essential micronutrient for plants; therefore maintenance of its homeostasis is required for normal plant physiology. One of the key components of  $Zn^{2+}$ homeostasis in plants involves the  $Zn^{2+}$ -ATPases. Although homologous ATPases have been found in bacteria, these have not been identified in other eukaryotes. Interestingly, plants have more than one (usually two or three)  $Zn^{2+}$ -ATPases. However, their tissue and cellular distribution patterns seem to be different indicating that each might have distinct physiological roles. In order to understand the functional characteristics and possible physiological roles of plant  $Zn^{2+}$ -ATPases, we studied *A. thaliana* HMA2.

Although the metal specificity of some plant  $Zn^{2+}$ -ATPases has been determined by bacterial or yeast complementation assays and metal uptake studies (Gravot et al., 2004; Mills et al., 2003) a direct metal dependent ATPase activity and direction of transport has not been established. We heterologously expressed HMA2 in yeast and measured the  $Zn^{2+}$  dependent ATPase activity in membranes (Eren, 2004). We showed that HMA2 is also activated by the non-physiological substrate  $Cd^{2+}$  with a similar  $V_{max}$ and  $K_{1/2}$  to  $Zn^{2+}$  (Eren, 2004). Other laboratories showed that HMA4 and HMA3,  $Zn^{2+}$ -ATPases from *A. thaliana*, confer resistance to  $Zn^{2+}/Cd^{2+}$  and  $Cd^{2+}/Pb^{2+}$ , respectively, when expressed in bacteria or yeast (Gravot et al., 2004; Mills et al., 2003). These results indicate that plant  $Zn^{2+}$ -ATPases have multiple substrate selectivity likely associated with the similar ionic radius and Lewis characteristics of these metals.

The proposed catalytic mechanism for the P-type ATPases requires binding of the metal from the cytoplasmic site of the membrane followed by its release to the extracellular space or lumen of the subcellular organelles (Post et al., 1972). However,

there have been a few reports suggesting influx of the metal by  $Cu^+$ -ATPases in bacteria (Odermatt et al., 1993; Tottey et al., 2001) that would require a coupling mechanism to drive phosphorylation/conformation of the enzyme in the absence of the metal. Determination of the direction of transport is one of the key elements for the determination of possible physiological role of these ATPases. Therefore we employed cell impermeable fluorescent Zn indicator, FluoZin-1, to follow Zn<sup>2+</sup> uptake into yeast vesicles. Our studies showed that HMA2 drives the Zn<sup>2+</sup> efflux from the cytoplasm (Eren, 2004).

*A. thaliana* has three Zn<sup>2+</sup>-ATPases. Multiple sequence analysis show that other plants including *O. sativa* and a hyperaccumulator plant *A. halleri* also have multiple putative Zn<sup>2+</sup>-ATPases (Williams, 2005, Eren unpublished). It is possible that these have distinct tissue distribution/subcellular location and different physiological roles. To determine the tissue distribution of HMA2, we analyzed HMA2 mRNA levels in seedlings, roots, leaves, stems and flowers by semi quantitative RT-PCR. Our results indicated that HMA2 expresses in all tested tissues with 50% higher levels in roots than in shoots. Another study by Hussain et al. (2004) showed that HMA2 is located in the plasma membrane and mainly expressed in the vasculature. The tissue distribution and subcellular localization of HMA2 seems different from HMA3 which is mainly expressed in roots and leaves and targeted to yeast vacuoles (Gravot et al., 2004). On the other hand, HMA2 and HMA4 seem to have similar distribution patterns since HMA4 is also expressed in all the tissues with significantly higher levels in roots, and is also located in the plasma membrane (Hussain, 2004).

To determine the possible physiological roles of HMA2 we analyzed the phenotypic characteristics of T-DNA insertion mutants. Although, *hma2* mutants did not show a visible phenotype from the wild type plants, we observed increased levels of  $Zn^{2+}$  or Cd<sup>2+</sup> in whole plants (Eren, 2004). This result indicated that HMA2 plays an important role in metal homeostasis mechanism. On the other hand, *hma2hma4* double mutants displayed a chlorotic and stunted phenotype that could be rescued by supplying excess Zn to the soil (Hussain, 2004). The *hma4* mutation alone seems to prevent translocation of Zn<sup>2+</sup> from roots to shoots indicating that HMA4 might be responsible for the loading of the xylem (Hussain, 2004). Together with the observed phenotype of the *hma2* mutant and the plasma membrane location of HMA2, mainly in the vasculature, suggested that this ATPase might have a central role in uploading Zn<sup>2+</sup> into the phoem.

Having established the key biochemical characteristics and *in vivo* functional role of HMA2, we turned our attention to peculiar structural features present in plant  $Zn^{2+}$ -ATPases. P<sub>1B</sub>-type ATPases have cytoplasmic regulatory MBDs in addition to TMBDs. Cu<sup>+</sup>-ATPases and some bacterial Zn<sup>2+</sup>-ATPases have typical N-MBDs with conserved CXXC signature sequences. The Cys have been shown to coordinate Cu<sup>+</sup> and Ag<sup>+</sup> in a linear geometry (Gitschier, 1998; Ralle, 2003). However, in the bacterial Zn<sup>2+</sup>-ATPase, ZntA, another residue, an Asp, is required to coordinate Zn<sup>2+</sup> together with the conserved Cys, probably in a tetrahedral geometry (Banci, 2002). These MBDs have been shown to regulate enzyme activity (Bal, 2001; Fan, 2002; Mana-Capelli, 2003; Mandal, 2003; Mitra, 2001; Tsivkovskii, 2001; Voskoboinik, 2001, 1999) and/or targeting (Forbes, 1999; Petris, 1996; Schaefer, 1999; Strausak and J., 1999). Plant Zn<sup>2+</sup>-ATPases have distinct sequences in both N- and C-termini that differentiate them from Cu<sup>+</sup>-ATPases and their bacterial homologues. Distinct from other  $P_{1B}$ -type ATPases, plant  $Zn^{2+}$ -ATPases have long C-terminus sequences rich in CC dipeptides and His repeats. Studies based on functional complementation approaches have provided conflicting results on the roles of putative C-MBDs of  $Zn^{2+}$ -ATPases. Truncation of the His-rich stretch (the last 16 amino acids of the C-terminus) of HMA4 impaired the enzyme's ability to complement *ycfl* ( $Cd^{2+}$ -sensitive) and *zrcl* ( $Zn^{2+}$ -sensitive) yeasts in the presence of excess amounts of  $Cd^{2+}$  or  $Zn^{2+}$  (Verret, 2005). On the other hand, truncation of the whole C-terminus of HMA4 did not affect the ability of HMA4 to confer Cd<sup>2+</sup> resistance to *vcf1* (Mills, 2005). In order to determine if the HMA2 C-terminus is a novel regulatory metal binding domain, we analyzed its metal binding properties and probable function. Our studies showed that the HMA2 C-terminus is a novel regulatory metal binding domain (Eren et al., 2006). We showed that removal of the C-terminus (C-MBD) of HMA2 leads to a 43% reduction in the enzyme turnover without significantly affecting the  $Zn^{2+} K_{1/2}$  suggesting this domain regulates enzyme activity probably by affecting the rate limiting step (Eren et al., 2006). Metal binding studies showed that  $Zn^{2+}$  binds to C-MBD with a stoichiometry of three (3 Zn/MBD) and is primarily coordinated by His in at least two of these sites with a tetrahedral geometry (Eren et al., 2006). Zn K-edge XAS of Zn-CMBD showed that in a single site  $Zn^{2+}$  is likely coordinated by a single Cys and three His residues suggesting that the  $Zn^{2+}$  binding sites are not equivalent.

All plant  $Zn^{2+}$ -ATPases lack the typical CXXC NMBDs. Instead, they have conserved CCXXE sequences. The two Cys and the acidic residue Glu contain side chains that can coordinate the metals. It is possible that the different arrangement of Cys from that of Cu<sup>+</sup>-ATPases might contribute to differential metal selectivity of these putative metal binding domains. To determine if plants have regulatory N-terminus metal binding domains with unique metal binding coordination, we studied the HMA2 N-terminus and showed that this is also a regulatory domain. Truncation of HMA2 N-terminus (N-MBD) leads to a  $\approx$  50% decrease in enzyme activity without altering metal binding affinity. Interestingly, truncation of both N-MBD and C-MBD also results in a  $\approx$  50% decrease suggesting that these domains might function in a coordinately. Mutation of the conserved Cys (Cys17Ala and Cys18A) or the Glu (Glu21Ala/Cys) in HMA2 N-MBD completely inhibited Zn<sup>2+</sup> binding to N-MBD suggesting that all these are involved in metal coordination. Mutations of these residues in HMA2 protein resulted in a reduction ( $\approx$ 25-50%) in the enzyme activity suggesting that the regulation of HMA2 by N-MBD is related to Zn<sup>2+</sup> binding to this domain.

In summary, we showed that *A. thaliana* HMA2 is a  $Zn^{2+}$ -ATPase with two novel regulatory cytoplasmic metal binding domains. This enzyme also appears to be responsible for  $Zn^{2+}$  loading into the phloem.

## **6. FUTURE DIRECTIONS**

In the years spent in Dr. Argüello's lab I have been able to explore many aspects of HMA2 structure and function. However, the objective of thesis is not only answering a number of questions regarding a certain topic, but also to open new lines of research to explore. In this regard, future research on the function and structure function of HMA2 should address these key questions:

How is  $Zn^{2+}$  delivered to HMA2? A number of authors have repeatedly pointed out that in the cytoplasm of living organisms there is no free metal. This observation led to the search of those molecules which deliver metals to the different metalloproteins, named metallochaperones. Cu<sup>+</sup>-metallochaperones have been well-characterized, as well as their interaction with Cu<sup>+</sup>-ATPases. However, a putative Zn<sup>2+</sup>-metallochaperone has not yet been identified. Since the cytoplasmic free metal concentrations is extremely low (less than one free metal per cell), it is thought that Zn<sup>2+</sup>-chaperones should also exist. In this context, HMA2 might be used as a bait to identify the protein that donates Zn<sup>2+</sup>, either by yeast-two hybrid system, co-immunoprecipitation, or affinity purification.

How do the different domains of HMA2 interact? In this study we showed that removing the N-, the C-MBD or both has the same effect on overall transport activity. These results strongly suggest that both domains act together. Site directed mutagenesis, FRET techniques, and X-ray crystallography would be useful to identify the characteristics of this interaction.

What is the effect of HMA2 on plant metal homeostasis? *hma2 A. thaliana* does not have a distinct phenotype. How do the other metal transporters compensate for the

loss of function? Transcriptome profiling of *hma2* and comparison to wild type plants by means of microarrays and quantitative RT-PCR will shed some light in this aspect.

The answers of these questions will enable us to enhance our knowledge about plant  $Zn^{2+}$ -ATPases, one of the key components of plant metal homeostasis. Understanding these mechanisms will eventually lead us to design plants that would contain elevated levels of essential heavy metals in their edible parts as a solution to heavy metal deficiency of some human populations; or to design plants that would be used for phytoremediation of heavy metal contaminated areas.

## REFERENCES

- Abdel-Ghany SE, Muller-Moule P, Niyogi KK, Pilon M, Shikanai T (2005) Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17: 1233-1251
- Afanes'ev, IB, Suslova, TB, Cheremisina, ZP, Abramova, NE, Korkina LG (1995) Study of antioxidant properties of metal aspartates. Analyst 120: 859-62.
- Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK,
  Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E,
  Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N,
  Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, AguilarHenonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden
  D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional
  mutagenesis of Arabidopsis thaliana. Science 301: 653-657
- Andrade MA, Chacón, P, Merelo, JJ, Morán, F (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. Protein Eng. 6: 383-390
- Andrés-Colás N, Sancenon V, Rodríguez-Navarro S, Mayo S, Thiele DJ, Ecker JR, Puig
  S, Peňarrubia L (2006) The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots.
  Plant J 45: 225-236
- Argüello JM (2003) Identification of ion selectivity determinants in heavy metal transport P1B-type ATPases. J Membr Biol 195: 93-108

- Argüello JM, Kaplan JH (1994) Glutamate 779, an intramembrane carboxyl, is essential for monovalent cation binding by the Na,K-ATPase. J Biol Chem 269: 6892-6899
- Argüello JM, Lingrel JB (1995) Substitutions of serine 775 in the alpha subunit of the Na,K-ATPase selectively disrupt K+ high affinity activation without affecting Na+ interaction. J Biol Chem 270: 22764-22771
- Argüello JM, Peluffo RD, Feng J, Lingrel JB, Berlin JR (1996) Substitution of glutamic 779 with alanine in the Na,K-ATPase alpha subunit removes voltage dependence of ion transport. J Biol Chem 271: 24610-24616
- Argüello JM, Whitis J, Cheung MC, Lingrel JB (1999) Functional role of oxygencontaining residues in the fifth transmembrane segment of the Na,K-ATPase alpha subunit. Arch Biochem Biophys 364: 254-263
- Arnesano F, Banci, L, Bertini, I, Ciofi-Baffoni, S, Molteni, E, Huffman, DL, and O'Halloran, TV (2002) Metallochaperones and metal-transporting ATPases: a comperative analysis of sequences and structures. Genome Res. 12: 255-271
- Arnesano F, Banci, L, Bertini, I, Huffman, DL, and O'Halloran, TV (2001) Solution structure of the Cu(I) and Apo forms of the yeast metallochaperone, Atx1. Biochemistry 40: 1528-1539
- Auld DS (2001) Zinc coordination sphere in biochemical zinc sites. BioMetals 14: 271-313
- Axelsen KB, and Palmgren, MG (1998) Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 46: 84-101
- Axelsen KB, and Palmgren, MG (2001) Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol 126: 696-706

- Baker-Austin C, Dopson M, Wexler M, Sawers RG, Bond PL (2005) Molecular insight into extreme copper resistance in the extremophilic archaeon 'Ferroplasma acidarmanus' Fer1. Microbiology 151: 2637-2646
- Bal N, Mintz, E, Guillain, F, and Catty, P (2001) A possible regulatory role for the metal metal-binding domain of CadA, the *Listeria monocytogenes* Cd<sup>2+</sup>-ATPase. FEBS Letters 506: 249-252
- Bal N, Wu, CC, Catty, P, Guillain, F, and Mintz, E (2003) Cd<sup>2+</sup> and the N-terminal metal-binding domain protect putative membranous CPC motif of the Cd<sup>2+</sup>-ATPase of *Listeria monocytogenes*. Biochem. J. 369: 681-685
- Balamurugan K, Schaffner W (2006) Copper homeostasis in eukaryotes: teetering on a tightrope. Biochim Biophys Acta 1763: 737-746
- Banci L, Bertini I, Ciofi-Baffoni S, Chasapis CT, Hadjiliadis N, Rosato A (2005) An NMR study of the interaction between human copper(I) chaperone and the second and fifth metal-binding domains of the Menkes protein, Febs J 272: 865-871.
- Banci L, Bertini, I, Ciofi-Baffoni, S, Finney, LA, Outten, CE, and O'Halloran, TV (2002)
  A new Zinc-protein coordination site in intracellular metal trafficking: solution structure of the apo and Zn(II) forms of ZntA (46-118). J Mol Biol 323: 883-897
- Banci L, Bertini, I, Ciofi-Baffoni, S, Huffman, DL, and O'Halloran, TV (2001) Solution structure of the yeast copper transporter domain Ccc2a in the Apo and Cu(I)loaded States. J. Biol. Chem. 276: 8415-8426
- Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A,
  Marshall M, Moxon S, Sonnhammer EL, Studholme DJ, Yeats C, Eddy SR
  (2004) The Pfam protein families database. Nucleic Acids Res 32: D138-141

- Baxter I, Tchieu J, Sussman MR, Boutry M, Palmgren MG, Gribskov M, Harper JF, Axelsen KB (2003) Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. Plant Physiol 132: 618-628
- Bissig KD, Wunderli-Ye H, Duda PW, Solioz M (2001) Structure-function analysis of purified Enterococcus hirae CopB copper ATPase: effect of Menkes/Wilson disease mutation homologues. Biochem J 357: 217-223
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254
- Buer CS, Masle J, Wasteneys GO (2000) Growth conditions modulate root-wave phenotypes in Arabidopsis. Plant Cell Physiol 41: 1164-1170
- Bulaj G, Kortemme, T., Goldenberg, D.P. (1998) Ionization-reactivity relationships for cysteine thiols in polypeptides. Biochemistry 37: 8965-8972
- Bull PC, and Cox, D.V. (1994) Wilson disease and Menkes disease: new handles on heavy-metal transport. Trends Genet 10: 246-252
- Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW (1993) The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat Genet 5: 327-337
- Callahan DL, Baker AJ, Kolev SD, Wedd AG (2006) Metal ion ligands in hyperaccumulating plants. J Biol Inorg Chem 11: 2-12
- Camakaris J, Petris, MJ, Bailey, L, Shen, P, Lockhart, P, Glower, TW, Barcroft, C, Patton, J, and Mercer, JF (1995) Gene amplification of the Menkes (MNK;

ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. Hum Mol Genet 4: 2117-2123

- Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran TV, Mondragon A (2003) Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science 301: 1383-1387
- Clark-Baldwin K, Tierney, DL, Govindaswamy, N, Gruff, ES, Kim, C, Berg, J, Koch, SA, and Penner-Hahn, JE (1998) The limitations of X-ray absorption spectroscopy for determining the structure of zinc sites in proteins. When is a tetrathiolate not a tetrathiolate. J Am Chem Soc 120: 8401-8409
- Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212: 475-486
- Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53: 159-182
- Colangelo EP, Guerinot ML (2006) Put the metal to the petal: metal uptake and transport throughout plants. Curr Opin Plant Biol 9: 322-330
- Corwin DTaK, S.A. (1988) Crystal structures of Zn(SR)<sub>2</sub> complexes: structural models for the proposed [Zn(cys-S)<sub>2</sub>(his)<sub>2</sub>] center in transcription factor IIIA and related nucleic acid binding proteins. Inorg Chem 27: 493-496
- DiDonato M, Hsu, H.F., Narindrasorasak, S., Que, L. Jr., and Sarkar, B. (2000) Copperinduced conformational changes in the N-terminal tomain of the Wilson Disease copper-transporting ATPase. Biochemistry 39: 1890-1896
- DiDonato M, Narindrasorasak, S., Forbes, J.R., Cox, D.W., and Sarkar, B. (1997) Expression, purification, and metal binding properties of the N-terminal domain

from the Wilson disease putative copper-transporting ATPase (ATP7B). J Biol Chem 272: 33279-33282

- DiDonato M, Zhang, J., Que, L., Jr., and Sarkar, B. (2002) Zinc binding to the NH<sub>2</sub>terminal domain of the Wilson disease copper-transporting ATPase: implications for in vivo metal ion-mediated regulation of ATPase activity. J. Biol. Chem. 277: 13409-13414
- Dmitriev O, Tsivkovskii R, Abildgaard F, Morgan CT, Markley JL, Lutsenko S (2006) Solution structure of the N-domain of Wilson disease protein: distinct nucleotidebinding environment and effects of disease mutations. Proc Natl Acad Sci U S A 103: 5302-5307
- Dutta SJ, Liu J, Hou Z, Mitra B (2006) Conserved aspartic acid 714 in transmembrane segment 8 of the ZntA subgroup of P1B-type ATPases is a metal-binding residue.
   Biochemistry 45: 5923-5931
- Eng BH, Guerinot, M.L., Eide, D., Saier, M.H., Jr. (1998) Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. J Membr Biol 166: 1-7
- Eren E, and Argüello, J.M. (2004) Arabidopsis HMA2, a divalent heavy metaltransporting P<sub>1B</sub>-type ATPase, is involved in cytoplasmic Zn<sup>+2</sup> homeostasis. Plant Physiol 136: 3712-3723
- Eren E, Kennedy DC, Maroney MJ, Arguello JM (2006) A Novel Regulatory Metal Binding Domain Is Present in the C Terminus of Arabidopsis Zn2+-ATPase HMA2. J Biol Chem 281: 33881-33891

- Fan B, and Rosen, B.P. (2002) Biochemical characterization of CopA, the *Escherichia coli* Cu(I)-translocating P-type ATPase. J Biol Chem 277: 46987-46992
- Forbes JR, Hsi, G., and Cox, D. W. (1999) Role of the copper binding domain in the copper transport function of ATP7B, the P-type ATPase defective in Wilson disease. J Biol Chem 274: 12408-12413
- Fox TC, Guerinot ML (1998) Molecular Biology of Cation Transport in Plants. Annu Rev Plant Physiol Plant Mol Biol 49: 669-696
- Fraga CG (2005) Relevance, essentiality and toxicity of trace elements in human health. Mol Aspects Med 26: 235-244
- Fraústro da Silva JJR, Williams RJP (2001) The Biological Chemistry of the Elements, Ed2. Oxford Unviversity Press, New York.
  - Fu D, Beeler TJ, Dunn TM (1995) Sequence, mapping and disruption of CCC2, a gene that cross-complements the Ca(2+)-sensitive phenotype of csg1 mutants and encodes a P-type ATPase belonging to the Cu(2+)-ATPase subfamily. Yeast 11: 283-292
  - Gaetke LM, Chow CK (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189: 147-163
  - Gee KR, Zhou ZL, Ton-That D, Sensi SL, Weiss JH (2002) Measuring zinc in living cells. A new generation of sensitive and selective fluorescent probes. Cell Calcium 31: 245-251

- Giedroc DP, Keating, K.M., Williams, K.R., Konigsberg, W.H., and Coleman, J.E.(1986) Gene 32 protein, the single stranded DNA binding protein from bacteriophage T4, is a zinc metalloprotein. Proc. Natl. Acad. Sci. 83: 8452-8456
- Gitschier J, Moffat, B., Reilly, D., Wood, W.I., and Fairbrother, W.J. (1998) Solution structure of the fourth metal-binding domain from the Menkes coppertransporting ATPase. Nat Struct Biol 5: 47-54
- Gomes E, Jakobsen MK, Axelsen KB, Geisler M, Palmgren MG (2000) Chilling tolerance in Arabidopsis involves ALA1, a member of a new family of putative aminophospholipid translocases. Plant Cell 12: 2441-2454
- Goodyer I, Jones E, Monaco A, Francis M (1999) Characterization of the Menkes protein copper-binding domains and their role in copper-induced protein relocalization. Hum Mol Genet 8: 1473-1478
- Goto JJ, Zhu, H., Sanchez, R.J., Nersissian, A., Gralla, E.B., and Valentine J.S. (2000)
   Loss of *in Vitro* Metal Ion Binding Specificity in Mutant Copper-Zinc Superoxide
   Dismutases Associated with Familial Amyotrophic Lateral Sclerosis. J. Biol.
   Chem. 275: 1007-1014
- Gravot A, Lieutaud A, Verret F, Auroy P, Vavasseur A, Richaud P (2004) AtHMA3, a plant P1B-ATPase, functions as a Cd/Pb transporter in yeast. FEBS Lett 561: 22-28
- Guerinot ML (2000) The ZIP family of metal transporters. Biochim Biophys Acta 1465: 190-198
- Guerinot ML, Eide D (1999) Zeroing in on zinc uptake in yeast and plants. Curr Opin Plant Biol 2: 244-249

- Guo J, Giedroc, D.P. (1997) Zinc site redesign in T4 gene 32 protein: structure and stability of Co(II) complexes formed by wild-type and metal ligand substitution mutants. Biochemistry 36: 730-742
- Guo Y, Nyasae L, Braiterman LT, Hubbard AL (2005) NH2-terminal signals in ATP7B
   Cu-ATPase mediate its Cu-dependent anterograde traffic in polarized hepatic
   cells. Am J Physiol Gastrointest Liver Physiol 289: G904-916
- Hacisalihoglu G, Hart JJ, Wang YH, Cakmak I, Kochian LV (2003) Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. Plant Physiol 131: 595-602
- Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53: 1-11
- Hamza I, Schaefer, M., Klamp, L.W., Gitlin, J.D. (1999) Interaction of the copper chaperone HAH1 with the Wison disease protein is essential for copper homeostasis. Proc Natl Acad Sci 96: 13363-13368
- Harrison MD, Meier, S., and Dameron, C.T. (1999) Characterisation of copper-binding to the second sub-domain of the Menkes protein ATPase (MNKr2). Biochim Biophys Acta 1453: 254-260
- Haupt M, Bramkamp M, Coles M, Altendorf K, Kessler H (2004) Inter-domain motions of the N-domain of the KdpFABC complex, a P-type ATPase, are not driven by ATP-induced conformational changes. J Mol Biol 342: 1547-1558
- He ZL, Yang XE, Stoffella PJ (2005) Trace elements in agroecosystems and impacts on the environment. J Trace Elem Med Biol 19: 125-140

- Himelblau E, Mira H, Lin SJ, Culotta VC, Penarrubia L, Amasino RM (1998)Identification of a functional homolog of the yeast copper homeostasis geneATX1 from Arabidopsis. Plant Physiol 117: 1227-1234
- Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97: 383-393
- Hou Z, Mitra, B. (2003) The metal specificity and selectivity of ZntA from Escherichia coli using the acylphosphate intermediate. J Biol Chem 278: 28455–28461
- Hou Z, Narindrasorasak S, Bhushan B, Sarkar B, Mitra B (2001) Functional Analysis of Chimeric Proteins of the Wilson Cu(I)-ATPase (ATP7B) and ZntA, a Pb(II)/Zn(II)/Cd(II)-ATPase from Escherichia coli. J Biol Chem 276: 40858-40863
- Huffman DL, and O'Halloran, T.V. (2000) Energetics of copper trafficking between the Atx1 metallochaperone and the intracellular copper transporter, Ccc2. J Biol Chem 275: 18611-18614
- Hung IH, Suzuki M, Yamaguchi Y, Yuan DS, Klausner RD, Gitlin JD (1997)
  Biochemical characterization of the Wilson disease protein and functional expression in the yeast Saccharomyces cerevisiae. J Biol Chem 272: 21461-21466
- Hussain D, Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper., J.F., and Cobbett, C.S. (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in arabidopsis. Plant Cell 16: 1327-1339

- Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153: 163-168
- Jensen PY, Bonander, N., Máller, L.B., and Farver, O., (1999) Cooperative binding of copper(I) to the metal binding domains in Menkes disease protein. Biochim Biophys Acta 1434: 103-113
- Kahn D, David M, Domergue O, Daveran ML, Ghai J, Hirsch PR, Batut J (1989)Rhizobium meliloti fixGHI sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J Bacteriol 171: 929-939
- Karlin S, and Zhu, Z.Y. (1997) Classification of mononuclear zinc metal sites in protein structures. Proc Natl Acad Sci 94: 14231-14236
- Kuntzweiler TA, Arguello JM, Lingrel JB (1996) Asp804 and Asp808 in the transmembrane domain of the Na,K-ATPase alpha subunit are cation coordinating residues. J Biol Chem 271: 29682-29687
- La Fontaine S, Firth SD, Lockhart PJ, Brooks H, Camakaris J, Mercer JF (1999) Functional analysis of the Menkes protein (MNK) expressed from a cDNA construct. Adv Exp Med Biol 448: 67-82
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685
- Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100: 95-97
- Larin D, Mekios, C., Das, K., Ross, B., Yang, A.S., and Gilliam, T.C. (1999) Characterization of the interaction between the Wilson and Menkes disease

proteins and the cytoplasmic copper chaperone, HAH1p. J Biol Chem 274: 28497-28504

- Lee J, Bae H, Jeong J, Lee JY, Yang YY, Hwang I, Martinoia E, Lee Y (2003) Functional expression of a bacterial heavy metal transporter in Arabidopsis enhances resistance to and decreases uptake of heavy metals. Plant Physiol 133: 589-596
- Li ZS, Lu Y-P, Zhen R-G, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci USA 94: 42-47
- Liu J, Dutta, S.J., Stemmler, A.J., and Mitra, B. (2006) Metal-binding affinity of the transmembrane site in ZntA: Implications for metal selectivity. Biochemistry 45: 763-772
- Liu J, Stemmler, A.J., Fatima, J., and Mitra, B. (2005) Metal-binding characteristics of the amino-terminal domain of ZntA: binding of lead is different compared to cadmium and zinc. Biochemistry 44: 5159-5167
- Liu SX, Fabisiak JP, Tyurin VA, Borisenko GG, Pitt BR, Lazo JS, Kagan VE (2000) Reconstitution of apo-superoxide dismutase by nitric oxide-induced copper transfer from metallothioneins. Chem Res Toxicol 13: 922-931
- Lobley A, Whitmore, L. and Wallace, B.A. (2002) DICHROWEB: an intreactive website for the analysis of protein secondary structure from circular dichroism spectra. Bioinformatics 18: 211-212

- Lowe J, Vieyra A, Catty P, Guillain F, Mintz E, Cuillel M (2004) A mutational study in the transmembrane domain of Ccc2p, the yeast Cu(I)-ATPase, shows different roles for each Cys-Pro-Cys cysteine. J Biol Chem 279: 25986-25994
- Lutsenko S, and Petris, M.J. (2003) Function and regulation of the mammalian coppertransporting ATPases: insights from biochemical and cell biological approaches. J Membr Biol 192: 1-12
- Lutsenko S, and Kaplan, J.H. (1995) Organization of P-type ATPases: significance of structural diversity. Biochemistry 34: 15607-15613
- Lutsenko S, Petrukhin, K., Cooper, M.J., Gilliam, C.T., and Kaplan, J.H. (1997) Nterminal domains of human copper-transporting adenosine triphosphatases (the Wilson's and Menkes disease proteins) bind copper selectively in vivo and in vitro with stoichiometry of one copper per metal-binding repeat. J Biol Chem 272: 18939-18944
- Lutsenko S, Tsivkovskii R, Walker JM (2003) Functional properties of the human copper-transporting ATPase ATP7B (the Wilson's disease protein) and regulation by metallochaperone Atox1. Ann N Y Acad Sci 986: 204-211
- MacLennan DH, Rice WJ, Odermatt A, Green NM (1998) Structure-function relationships in the Ca(2+)-binding and translocation domain of SERCA1: physiological correlates in Brody disease. Acta Physiol Scand Suppl 643: 55-67
- Mana-Capelli S, Mandal, A.K., and Argüello, J.M. (2003) *Archaeoglobus fulgidus* CopB is a thermophilic Cu<sup>2+</sup>-ATPase: Functional role of its histidine-rich N-terminal metal binding domain. J Biol Chem 278: 40534-40541

- Mandal AK, and Argüello, J.M. (2003) Functional roles of metal binding domains of the *Archaeglobus fulgidus* Cu<sup>+</sup>-ATPase CopA. Biochemistry 42: 11040-11047
- Mandal AK, Cheung WD, Arguello JM (2002) Characterization of a thermophilic P-type Ag+/Cu+-ATPase from the extremophile Archaeoglobus fulgidus. J Biol Chem 277: 7201-7208
- Mandal AK, Yang, Y., Kertesz, T.M., and Argüello, J.M. (2004) Identification of the transmembrane metal binding site in Cu<sup>+</sup>-transporting P1B-type ATPases. J Biol Chem 279: 54802-54807
- Marschner H (1995) Mineral Nutrition of Higher Plants. Ed 2, Academic Press, London
- Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A,
  Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE,
  Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter
  families of Arabidopsis. Plant Physiol 126: 1646-1667
- Melchers K, Weitzenegger T, Buhmann A, Steinhilber W, Sachs G, Schafer KP (1996) Cloning and membrane topology of a P type ATPase from Helicobacter pylori. J Biol Chem 271: 446-457
- Mills RF, Francini, A., Ferreira da Rocha, P.S.C., Baccarani, P.J., Aylett M., Krijger, G.C., and Williams L.E. (2005) The plant P<sub>1B</sub>-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. FEBS Letters 579: 783-791
- Mills RF, Krijger GC, Baccarini PJ, Hall JL, Williams LE (2003) Functional expression of AtHMA4, a P1B-type ATPase of the Zn/Co/Cd/Pb subclass. Plant J 35: 164-176

- Mitra B, and Sharma, R. (2001) The cysteine-rich amino-terminal domain of ZntA, a Pb(II)/Zn(II)/Cd(II)-translocating ATPase from *Escherichia coli*, is not essential for its function. Biochemistry 40: 7694-7699
- Møller JVJ, B., and le Marie, M. (1996) Structural organization, ion transport, and energy transduction of P-type ATPases. Biochim Biophys Acta 1286: 1-51
- Morgan CT, Tsivkovskii R, Kosinsky YA, Efremov RG, Lutsenko S (2004) The distinct functional properties of the nucleotide-binding domain of ATP7B, the human copper-transporting ATPase: analysis of the Wilson disease mutations E1064A, H1069Q, R1151H, and C1104F. J Biol Chem 279: 36363-36371
- Myari A, Hadjiliadis N., Fatemi N., and Sarkar, B. (2004) Copper (I) interaction with model peptides of WD6 and TM6 domains of Wilson ATPase: regulatory and mechanistic implications. J Inorg Chem 98: 1483-1494
- Nucifora G, Chu L, Misra TK, Silver S (1989) Cadmium resistance from Staphylococcus aureus plasmid pI258 cadA gene results from a cadmium-efflux ATPase. Proc Natl Acad Sci U S A 86: 3544-3548
- Odermatt A, Suter H, Krapf R, Solioz M (1993) Primary structure of two P-type ATPases involved in copper homeostasis in Enterococcus hirae. J Biol Chem 268: 12775-12779
- O'Halloran TV, Culotta VC (2000) Metallochaperones, an Intracellular Shuttle Service for Metal Ions. J Biol Chem 275: 25057-25060
- Okkeri J, Bencomo E, Pietila M, Haltia T (2002) Introducing Wilson disease mutations into the zinc-transporting P-type ATPase of Escherichia coli: The mutation P634L

in the 'hinge' motif (GDGXNDXP) perturbs the formation of the E2 P state. Eur J Biochem 269: 1579-1586

- Okkeri J, Haltia T (1999) Expression and mutagenesis of ZntA, a zinc-transporting Ptype ATPase from Escherichia coli. Biochemistry 38: 14109-14116
- Okkeri J, Laakkonen L, Haltia T (2004) The nucleotide-binding domain of the Zn2+transporting P-type ATPase from Escherichia coli carries a glycine motif that may be involved in binding of ATP. Biochem J 377: 95-105
- Olesen C, Sorensen TL, Nielsen RC, Møller JV, Nissen P (2004) Dephosphorylation of the calcium pump coupled to counterion occlusion. Science 306: 2251-2255
- Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of Metal-binding Peptides by HMT1, A Fission Yeast ABC-type Vacuolar Membrane Protein. J Biol Chem 270: 4721-4728
- Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292: 2488-2492
- Padden KMK, J.F.; Trafford, K.T.; Yap, G.P.A.; Rheingold, A.H.; Borovik, A.S.; and Scarrow, R.C. (2001) Probing the structure of immobilized metal sites in porous organic hosts by X-ray Absorption Spectroscopy. Chem Mater 13: 4305-4313
- Paulsen IT, Saier, M.H., Jr. (1997) A novel family of ubiquitous heavy metal ion transport proteins. J Membr Biol 156: 99-103
- Pedersen PA, Nielsen JM, Rasmussen JH, Jørgensen PL (1998) Contribution to Tl+, K+, and Na+ binding of Asn776, Ser775, Thr774, Thr772, and Tyr771 in cytoplasmic part of fifth transmembrane segment in alpha-subunit of renal Na,K-ATPase. Biochemistry 37: 17818-17827

- Pedersen PA, Rasmussen JH, Nielsen JM, Jørgensen PL (1997) Identification of Asp804 and Asp808 as Na+ and K+ coordinating residues in alpha-subunit of renal Na,K-ATPase. FEBS Lett 400: 206-210
- Pedersen PL, Carafoli, E. (1987) Ion motive ATPases: I. Ubiquity, properties, and significance to cell function. Trends Biochem Sci 12: 146–150
- Pena MMO, Lee J, Thiele DJ (1999) A Delicate Balance: Homeostatic Control of Copper Uptake and Distribution. J. Nutr. 129: 1251-1260
- Pennela MA, Arunkumar, A.I., and Giedroc D.P. (2006) Individual metal ligands play distinct functional roles in the zinc sensor Staphylococcus aureus CzrA. J. Mol. Biol. 356: 1124-1136
- Petris MJ, Mercer, J.F., Culvenor, J.G., Lockhart, P., Gleeson, P.A., and Camakaris, J. (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. Embo J 15: 6084-6095
- Petrukhin K, Lutsenko S, Chernov I, Ross BM, Kaplan JH, Gilliam TC (1994) Characterization of the Wilson disease gene encoding a P-type copper transporting ATPase: genomic organization, alternative splicing, and structure/function predictions. Hum Mol Genet 3: 1647-1656
- Phung LT, Ajlani G, Haselkorn R (1994) P-type ATPase from the cyanobacterium Synechococcus 7942 related to the human Menkes and Wilson disease gene products. Proc Natl Acad Sci U S A 91: 9651-9654

- Portnoy ME, Rosenzweig AC, Rae T, Huffman DL, O'Halloran TV, Culotta VC (1999) Structure-Function Analyses of the ATX1 Metallochaperone. J. Biol. Chem. 274: 15041-15045
- Post RL, Hegyvary C, Kume S (1972) Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. J Biol Chem 247: 6530-6540
- Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284: 805-808
- Ralle M, Lutsenko, S., and Blackburn, N.J. (2003) The Menkes disease protein binds copper via novel 2-coordinate Cu<sup>+</sup>-cysteinates in the N-terminal domain. J Biol Chem 278: 23163-23170
- Rauser WE (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins. Cell Biochem Biophys 31: 19-48
- Rea P (1999) MRP subfamily ABC transporters from plants and yeast. J Exp Bot 50: 895–913
- Rensing C, Fan B, Sharma R, Mitra B, Rosen BP (2000) CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci U S A 97: 652-656
- Rensing C, Ghosh, M., and Rosen, B.P. (1999) Families of soft-metal-ion transporting ATPases. J Bacteriol 181: 5891-5897
- Rensing C, Mitra B, Rosen BP (1997) The zntA gene of Escherichia coli encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci U S A 94: 14326-14331

- Rensing C, Mitra, B., Rosen, B.P. (1998a) The zntA gene of Escherichia coli encodes a Zn(II)-translocating P-type ATPase. J Biol Chem 273: 3765–3770
- Rensing C, Mitra, B., Rosen, B.P. (1998b) A Zn(II)-translocating P-type ATPase from Proteus mirabilis. Biochem Cell Biol 76: 787–790
- Roberts VA, Fisher CL, Redford SM, McRee DE, Parge HE, Getzoff ED, Tainer JA (1991) Mechanism and atomic structure of superoxide dismutase. Free Radic Res Commun 12-13 Pt 1: 269-278
- Rosenzweig AC, Huffman, D.L., Hou, M.Y., Wernimont, A.K., Pufahl, R.A., and O'Halloran, T.V. (1999) Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. Structure 7: 605-617
- Rutherford JC, Cavet JS, Robinson NJ (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPxtype ATPase. J Biol Chem 274: 25827-25832
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sarkadi B, Enyedi A, Foldes-Papp Z, Gardos G (1986) Molecular characterization of the in situ red cell membrane calcium pump by limited proteolysis. J Biol Chem 261: 9552-9557
- Sazinsky MH, Agarwal S, Argüello JM, Rosenzweig AC (2006) Structure of the actuator domain from the Archaeoglobus fulgidus Cu(+)-ATPase. Biochemistry 45: 9949-9955

- Sazinsky MH, Mandal AK, Argüello JM, Rosenzweig AC (2006) Structure of the ATP binding domain from the Archaeoglobus fulgidus Cu+-ATPase. J Biol Chem 281: 11161-11166
- Schaefer M, Hopkins, R.G., Failla, M.L., and Gitlin, J.D. (1999) Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. Am J Physiol 276: G639-G646
- Seigneurin-Berny D, Gravot A, Auroy P, Mazard C, Kraut A, Finazzi G, Grunwald D, Rappaport F, Vavasseur A, Joyard J, Richaud P, Rolland N (2006) HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281: 2882-2892
- Sharma R, Rensing C, Rosen BP, Mitra B (2000) The ATP hydrolytic activity of purified ZntA, a Pb(II)/Cd(II)/Zn(II)-translocating ATPase from Escherichia coli. J Biol Chem 275: 3873-3878
- Shikanai T, Muller-Moule P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15: 1333-1346
- Solioz M, and Vulpe, C. (1996) CPx-type ATPases: a class of P-type ATPases that pump heavy metals. Trends Biochem Sci 21: 237-241
- Solioz M, Odermatt A (1995) Copper and silver transport by CopB-ATPase in membrane vesicles of Enterococcus hirae. J Biol Chem 270: 9217-9221
- Steele RA, and Opella, S.J. (1997) Structures of the reduced and mercury-bound forms of MerP, the periplasmic protein from the bacterial detoxification system. Biochemistry 36: 6885-6895

- Strausak D, Howie, M.K., Firth, S.D., Sclicksupp, A., Pipkorn, R., Multhaup, G., and Mercer, J.F. (2003) Kinetic analysis of the interaction of the copper chaperone Atox1 with the metal binding sites of the Menkes protein. J Biol Chem 278: 20821-20827
- Strausak D, La Fontaine, S., Hill, J., Firth, S. D., Lockhart, P., J. aM, J. F. (1999) The role of GMXCXXC metal binding sites in the copper-induced redistribution of the Menkes protein. J. Biol. Chem. 274: 11170-11177
- Tapiero H, Tew KD (2003) Trace elements in human physiology and pathology: zinc and metallothioneins. Biomed Pharmacother 57: 399-411
- Tapiero H, Townsend DM, Tew KD (2003) Trace elements in human physiology and pathology. Copper. Biomed Pharmacother 57: 386-398
- Tottey S, Rich PR, Rondet SA, Robinson NJ (2001) Two Menkes-type atpases supply copper for photosynthesis in Synechocystis PCC 6803. J Biol Chem 276: 19999-20004
- Toyoshima C, Inesi G (2004) Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 73: 269-292
- Toyoshima C, Nakasako M, Nomura H, Ogawa H (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 405: 647-655
- Toyoshima C, Nomura H (2002) Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 418: 605-611
- Toyoshima C, Nomura H, Tsuda T (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. Nature 432: 361-368

- Tsai KJ, Lin YF, Wong MD, Yang HH, Fu HL, Rosen BP (2002) Membrane topology of the p1258 CadA Cd(II)/Pb(II)/Zn(II)-translocating P-type ATPase. J Bioenerg Biomembr 34: 147-156
- Tsai KJ, Linet AL (1993) Formation of a phosphorylated enzyme intermediate by the cadA Cd(2+)-ATPase. Arch Biochem Biophys 305: 267-270
- Tsai KJ, Yoon KP, Lynn AR (1992) ATP-dependent cadmium transport by the cadA cadmium resistance determinant in everted membrane vesicles of Bacillus subtilis. J Bacteriol 174: 116-121
- Tsivkovskii R, Efremov RG, Lutsenko S (2003) The role of the invariant His-1069 in folding and function of the Wilson's disease protein, the human copper-transporting ATPase ATP7B. J Biol Chem 278: 13302-13308
- Tsivkovskii R, MacArthurs, B., and Lutsenko, S. (2001) The Lys(1010)-Lys(1325) fragment of the Wilson's disease protein binds nucleotides and interacts with the N-terminal domain of this protein in a copper-dependent manner. J Biol Chem 276: 2234-2242
- VanZile ML, Cosper, N.J., Scott, R.A., and Giedroc, D.P. (2000) The zinc metalloregulatory protein *Synechococcus* PCC7942 SmtB binds a single zinc ion per monomer with high affinity in a tetrahedral coordination geometry. Biochemistry 39: 11818-11829
- Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett 576: 306-312

- Verret F, Gravot, A., Auroy, P., Preveral, S., Frostier, C., Vavasseur, A., and Richaud, P. (2005) heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His<sub>11</sub> stretch. FEBS Letters 579: 1515-1522
- Vilsen B, Andersen JP (1998) Mutation to the glutamate in the fourth membrane segment of Na+,K+-ATPase and Ca2+-ATPase affects cation binding from both sides of the membrane and destabilizes the occluded enzyme forms. Biochemistry 37: 10961-10971
- Voskoboinik I, Brooks H, Smith S, Shen P, Camakaris J (1998) ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells. FEBS Lett 435: 178-182
- Voskoboinik I, Mar, J., Strausak, D., and Camakaris J. (2001) The regulation of catalytic activity of the Menkes copper-translocating P-type ATPase: Role of high affinity copper-binding sites. J Biol Chem 276: 28620-28627
- Voskoboinik I, Strausak, D., Greenough, M. Brooks, H., Petris, M., Smith, S., Mercer, J.F., and Camakaris, J. (1999) Functional analysis of the N-terminal CXXC metalbinding motifs in the human Menkes copper-transporting P-type ATPase expressed in cultured mammalian cells. J Biol Chem 274: 22008–22012
- Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 3: 7-13

- Walker JM, Tsivkovskii, R., and Lutsenko, S. (2002) Metallochaperone Atox1 transfers copper to the NH2-terminal domain of the wilson's disease protein and regulates its catalytic activity. J Biol Chem 277: 27953-27959
- Walkup GK, and Imperiali, B. (1997) Fluorescent chemosensors for divalent zinc based on zinc finger domains. Enhanced oxidative stability, metal binding affinity, and structural and functional characterization. J Am Chem Soc 119: 3443-3450
- Wernimont AK, Huffman, D.L., Lamb, A.L., O'Halloran, T.V., and Rosenzweig, A.C.(2000) Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. Nat Struct Biol 7: 766-771
- Wernimont AK, Yatsunyk, L.A., and Rosenzweig, A.C. (2004) Binding of copper(I) by the Wilson disease protein and its copper chaperone. J Biol Chem 279: 12269-12276
- Whitmore L, and Wallace, B.A. (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Research 32: W668-W673
- Williams LE, and Mills, R.F. (2005) P<sub>1B</sub>-ATPases-an ancient family of transition metal pumps with diverse function in plants. Trends Plant Sci 10: 491-502
- Williams LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. Biochim Biophys Acta 1465: 104-126
- Woeste KE, Kieber JJ (2000) A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell 12: 443-455

- Woolhouse HW (1983) Toxicity and tolerance in the responses of plants to metals. *In* OL
  Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology III.
  Responses to the Chemical and Biological Environment, Vol 12C. SpringerVerlag, Berlin
- Yoshimizu T, Omote H, Wakabayashi T, Sambongi Y, Futai M (1998) Essential Cys-Pro-Cys motif of Caenorhabditis elegans copper transport ATPase. Biosci Biotechnol Biochem 62: 1258-1260
- Yuan DS, Dancis A, Klausner RD (1997) Restriction of copper export in Saccharomyces cerevisiae to a late Golgi or post-Golgi compartment in the secretory pathway. J Biol Chem 272: 25787-25793
- Yuan DS, Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R.D. (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc Natl Acad Sci USA 92: 2632-2636