A Novel Regulatory Metal Binding Domain Is Present in the C Terminus of Arabidopsis Zn\(^{2+}\)-ATPase HMA2\(^*\)

Elif Eren\(^1\), David C. Kennedy\(^1\), Michael J. Maroney\(^1\), and José M. Argüello\(^1\)

From the \(^1\)Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, Massachusetts 01609 and the \(^2\)Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

HMA2 is a Zn\(^{2+}\)-ATPase from Arabidopsis thaliana. It contributes to the maintenance of metal homeostasis in cells by driving Zn\(^{2+}\) efflux. Distinct from P\(_{1B}\)-type ATPases, plant Zn\(^{2+}\)-ATPases have long C-terminal sequences rich in Cys and His. 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\(_T\) for enzyme activation. Characterization of the isolated HMA2 C terminus showed that this fragment binds three Zn\(^{2+}\) with high affinity (K\(_D\) = 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). Zn K-edge XAS of Zn-C-MBD in the presence of one equivalent of Zn\(^{2+}\) shows that the average zinc complex formed is composed of three His and one Cys residues. Upon the addition of two extra Zn\(^{2+}\) ions per C-MBD, these appear coordinated primarily by His residues thus, suggesting that the three Zn\(^{2+}\) binding domains might not be identical. Modification of His residues with diethyl pyrocarbonate 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 did not prevent Zn\(^{2+}\) binding to the HMA2 C terminus. Zn K-edge XAS of the Cys-alkylated protein was consistent with (N/O)\(_4\) coordination of the zinc 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.

P\(_{1B}\)-type ATPases, a subfamily of P-type ATPases, transport heavy metals (Ag\(^{+}\), Cu\(^{+}\), Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Pb\(^{2+}\), Co\(^{2+}\)) across biological membranes (1–3). These enzymes play critical roles in maintaining heavy metal homeostasis in organisms ranging from bacteria to humans (4–8). Plant genomes appear to contain multiple (eight or nine) genes encoding P\(_{1B}\)-ATPases with various metal selectivities (Zn\(^{2+}\)-ATPases, Cu\(^{+}\)-ATPases, and others with metal dependence is still to be determined) (3, 8, 9). Distinctly, only two Cu\(^{+}\)-ATPase isoforms are found in other eukaryotes (1–3). We recently characterized the functional role of Arabidopsis thaliana HMA2 (10). This Zn\(^{2+}\)-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\(^{2+}\) and Cd\(^{2+}\) levels (10). This observation along with the plasma membrane localization and strong expression in the plant vasculature suggests that HMA2 is responsible for Zn\(^{2+}\) uploading into the phloem (10, 11).

P\(_{1B}\)-type ATPases have 6–8 transmembrane fragments responsible for metal translocation and a large cytoplasmic loop involved in ATP binding and hydrolysis (1–3). 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 (3, 12). Most of these enzymes also have highly conserved N-terminal metal binding domains (N-MBDs)\(^2\) characterized by the CXXC sequences (3, 6, 7, 13). These Cys residues are responsible for metal coordination, and can bind both monovalent and divalent cations (Cu\(^{+}\), Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\)) (14–19). In Cu\(^{+}\)-ATPases, N-MBDs receive the metal from specific Cu\(^{+}\)-chaperones (20–26). 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 (27–33). Lutsenko and co-workers (34) have shown the Cu\(^{+}\)-dependent interaction of Wilson’s disease protein N-MBDs with the large ATP binding cytoplasmic loop. In our laboratory, we have observed that N-MBDs of Archaeoglobus fulgidus CopA, a Cu\(^{+}\)-ATPase, and CopB, a Cu\(^{2+}\)-ATPase with a His-rich N-MBD, control the turnover rate of these enzymes but do not affect metal binding to the transport site (32, 33). Specifically, the rate-limiting conformational change associated with metal release/dephosphorylation is affected by metal binding to N-MBDs (32, 33). Thus, N-MBDs, although not essential for activity, are key regulators.

\(^*\) This work was supported by National Science Foundation Grant MCM-0235165 (to J. M. A.) and National Institutes of Health Grant R01-GM061696 (to M. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^2\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3, two equations, and data analysis.

To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, Worcester Polytechnic Inst., 100 Institute Rd, Worcester, MA 01609. Tel.: 508-831-5326; Fax: 508-831-5933; E-mail: arguello@wpi.edu.

References

1. Armstrong, D. L., and M. J. Maroney. (2002) J. Biol. Chem. 277, 39919–39926.

2. The abbreviations used are: N-MBD, N-terminal metal binding domain; AAS, atomic absorption spectroscopy; 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; TCEP, Tris (2-carboxyethyl)phosphine; XAS, x-ray absorption spectroscopy; XANES, x-ray absorption near-edge structure; g.o.f., goodness of fit.
tors of enzyme function. In addition, studies of the human Cu\(^{+}\)-ATPases, Menkes and Wilson disease 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 (5, 35–37).

Many bacterial Zn\(^{2+}\)-ATPases seem to contain the typical CXXC N-MBDs (3, 29). It has been shown that in ZntA, Cys in the conserved GMDCCXX motif coordinate metal ions with high affinity (19, 38). Similar to Cu\(^{+}\)-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 (29, 39). 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 Cys-Cys repeat sequences and His residues. These Cys- and His-rich segments are uncommon among non-plant \(_{1\mu}\)-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\(^{2+}\)-ATPases putative C-MBDs. Truncation of the C terminus His-rich stretch (the last 16 amino acids of the C terminus) of \(A.\ thaliana\) HMA4 impaired the enzyme ability to complement \(\text{ycf}\) \(\text{f1}\) (Cd\(^{2+}\)-sensitive) and \(\text{zrc}\) \(\text{1}\) (Zn\(^{2+}\)-sensitive) yeasts in the presence of high Cd\(^{2+}\) or Zn\(^{2+}\) (40). 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 \(\text{ycf}\) \(\text{f1}\) yeast (41). 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.

**EXPERIMENTAL PROCEDURES**

**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 plasmid \(p\)PRIBA vector (10) using the oligonucleotides: 5'-\text{AGAGGTACCAATAATGGCGTCGAGGA}\ 3' and 5'-\text{CTGCAGGAGTTCCTATCAACAATTGTCGAGGA}\ 3'. Resulting cDNA was ligated into the KpnI and Xhol sites of \(p\)PRIBA vector (IBA, Göttingen, Germany). This vector introduces a Strep tag at the C-terminal end of the protein. HMA2-Strep tag cDNA was amplified using the oligonucleotides: 5'-\text{AGAGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGT-}

TTAAACTTTTTTCGACTG} and the HMA2-pPRIBA1 as a template. The amplicon was ligated into the KpnI and Pmel sites of the yeast expression vector \(p\)YES2/CT (Invitrogen). Constructs to express truncated HMA2 proteins were prepared: \(\Delta\)C-HMA2 cDNA encoding HMA2 lacking the 244 C-terminal amino acids, starting in Met and ending in Glu\(^{707}\), and \(\Delta\)N-C-HMA2 encoding HMA2 lacking the N-terminal first 75 amino acids and C-terminal 244 amino acids, starting from Val and ending in Glu\(^{707}\). \(\Delta\)C-HMA2 and \(\Delta\)N,C-HMA2 constructs were amplified from HMA2-pBAD/TOPO construct using the oligonucleotides: \(\Delta\)C-HMA2 5'-\text{AGAGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}.}

**Yeast Membrane Preparation—**Membranes from yeast were prepared as previously described (10, 30). Protein concentrations were measured in accordance to Bradford (42). SDS-PAGE was carried out in 10% acrylamide gels (43). Heterologous proteins in the membrane preparations were detected by electrophoretically 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 Alphalmager software (Alpha Innotech Corp., San Laendro, CA).

**ATPase Assay—**Metal-dependent ATPase activity determinations were performed as previously described (10) in a media containing 50 m\(\text{M}\) Tris, pH 7.5, 3 m\(\text{M}\) MgCl\(_2\), 3 m\(\text{M}\) ATP, 20 m\(\text{M}\) cysteine, 1 m\(\text{M}\) dithiothreitol, 0.5 mg ml\(^{-1}\) saponin, and 40 \(\mu\text{g}\) ml\(^{-1}\) protein (membrane preparation), at 30 °C. The concentrations of ZnCl\(_2\) or CdCl\(_2\) were varied as indicated in the figures. ATPase activity measured in the absence of metal was always \(<12.5\%\) of \(V_{\text{max}}\) 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 (10). Curves of ATPase activity versus metal concentration were fit to \(v = V_{\text{max}}L/(L + K_{\text{Is}})\), where \(L\) is the concentration of the variable ligand. The reported standard errors for \(V_{\text{max}}\) and \(K_{\text{Is}}\) are asymptotic standard errors reported by the fitting software (Kaleidograph, Synergy, Reading, PA).

**Cloning, Expression, and Purification of HMA2 C-MBD—**A cDNA coding for the last 244 amino acids of HMA2, from S.\text{Y}cog to Glu\(^{951}\) (C-MBD) was amplified by using the oligonucleotides: 5'-\text{GGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}\}

TTAAACTTTTTTCGACTG} and the HMA2-pPRIBA1 as a template. Resulting amplicon was cloned into the KpnI and Pmel sites of the yeast expression vector \(p\)YES2/CT (Invitrogen). Constructs to express truncated HMA2 proteins were prepared: \(\Delta\)C-HMA2 cDNA encoding HMA2 lacking the 244 C-terminal amino acids, starting in Met and ending in Glu\(^{707}\), and \(\Delta\)N-C-HMA2 encoding HMA2 lacking the N-terminal first 75 amino acids and C-terminal 244 amino acids, starting from Val and ending in Glu\(^{707}\). \(\Delta\)C-HMA2 and \(\Delta\)N,C-HMA2 constructs were amplified from HMA2-pBAD/TOPO construct using the oligonucleotides: \(\Delta\)C-HMA2 5'-\text{AGAGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}\ and 3'-\text{GGGTACCAATAATGGCGTCGAGGA}.}

**REFERENCES**

1. Bozec, C., & Tissier, C. (2004) J. Biol. Chem. 279, 49119–49129.
2. Bozec, C., & Tissier, C. (2005) J. Biol. Chem. 280, 20607–20614.
3. Bozec, C., & Tissier, C. (2006) J. Biol. Chem. 281, 39949–39959.
4. Bozec, C., & Tissier, C. (2007) J. Biol. Chem. 282, 14449–14457.
5. Bozec, C., & Tissier, C. (2008) J. Biol. Chem. 283, 27634–27643.
6. Bozec, C., & Tissier, C. (2009) J. Biol. Chem. 284, 17658–17666.
7. Bozec, C., & Tissier, C. (2010) J. Biol. Chem. 285, 15343–15352.
8. Bozec, C., & Tissier, C. (2011) J. Biol. Chem. 286, 25552–25560.
9. Bozec, C., & Tissier, C. (2012) J. Biol. Chem. 287, 17334–17342.
10. Bozec, C., & Tissier, C. (2013) J. Biol. Chem. 288, 9147–9155.
11. Bozec, C., & Tissier, C. (2014) J. Biol. Chem. 289, 17334–17342.
12. Bozec, C., & Tissier, C. (2015) J. Biol. Chem. 290, 17334–17342.
erichia coli BL21(DE3)pLysS cells were transformed with this vector. C-MBD expression was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside for 3 h. Cells were collected by centrifugation at 2500 \times 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 \times 30 s) and centrifuged at 10,000 \times g for 20 min. The resulting supernatant was collected and centrifuged at 110,000 \times g for 60 min. The supernatant was passed through Strep-Tactin Superflow column (IBA). The column was washed with five volumes of homogenization buffer, and the C-MBD was eluted with 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM TCEP, and 2.5 mM dethiobiotin. The protein was concentrated to 3 mg ml^{-1} using an Amicon Ultra-15 Centricron (Millipore, Billerica, MA) and stored in 100 mM Tris, pH 8.00, 150 mM NaCl, and 1 mM TCEP at \(-80^\circ C\). Routine protein concentration determinations were performed in accordance to Bradford (42).

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 hydrolyzed (Keck Facility, Yale University, New Haven, CT). Before metal binding stoichiometry determinations the storage buffer was hydrolyzed (Keck Facility, Yale University, New Haven, CT). Before metal binding stoichiometry determinations the storage buffer was hydrolyzed (Keck Facility, Yale University, New Haven, CT). Before metal binding stoichiometry determinations the storage buffer was hydrolyzed (Keck Facility, Yale University, New Haven, CT). Before metal binding stoichiometry determinations the storage buffer was hydrolyzed (Keck Facility, Yale University, New Haven, CT).

Metal Binding Stoichiometry Determinations—Metal contents were measured by atomic absorption spectroscopy (AAS) (AAAnalyst 300, PerkinElmer Life Sciences). 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^{2+}, Cd^{2+}, and Co^{2+}) 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 Centricron (Millipore) with 15 volumes 20 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM TCEP.

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 (38, 44). 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+} (38). An extinction coefficient of 29,900 M^{-1} cm^{-1} at 366 nm for metal-free mag-fura-2 and $K_i$ of 5.0 \times 10^7 M^{-2} was used in determinations of free mag-fura-2 and free Zn^{2+} (45). The metal-protein association constant ($K_a$) and the number of metal binding sites (n) in C-MBD, were calculated by fitting the data to $\nu = nK_a[Met]/(1 + K_a[Met])$, where $\nu$ is the ratio of moles of metal bound to total protein (46). As above, reported errors for $K_a$ 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 (47). 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^{-1} cm^{-1}).

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^{2+}, Co^{2+}, Cu^{2+}). 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 using the K2d analysis algorithm (48–50).

Carboxymethylation of Cysteines—C-MBD (1 mg/ml) was incubated with 10 mM dithiothreitol 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 iodoacetic acid (IAA) for 30 min at 25 °C in the dark. The protein was washed in an Amicon Ultra-15 Centricron (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 Centricron (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^{-1}) as previously described (51).

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 Centricron (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 sagittally 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^{2+} 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 \sim 9460–9860 eV and x-ray absorption fine structure (EXAFS) were collected from 9460–10640 eV. The primary vertical apertures was set to 0.3 mm for all samples. EXAFS data were analyzed using the program EXAFS123 (52) using parameters, including multiple scattering parameters for His imidazole ligands, using FEFF8. Details of the data analysis are provided under supplementary materials.
A. thaliana HMA2 C-MBD

**A. Extracellular**

- Numbers in white boxes indicate the position of transmembrane segments within the HMA2 sequence. C347, P637, C637, and P637 are conserved in all Zn\textsuperscript{2+}-ATPases (3).

- **B. Cytoplasm**

  - **MGDRPEFELGTSSSSLVIAEKLEDAAGMEAGLPPKISD**
  - **KHCKPNGCCGTQEKAMPKASDDSHSSGCCETKQKDNV**
  - **TVKKSSCCAEPLGDGHGDSGCGKDSOQPQHEVQVOQQ**
  - **SCHNPSGLDSSCGKSQQPOHQHELQQSCDKPSGLDGT**
  - **GPKHGSSTLVDGEAMDKVEKDLVNGFCSSPADLATSLS**
  - **KSDSHCKSCNSSRCRCHGSNCCRSYAKESCSHDDHDHITRAH**

**FIGURE 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. C347, PC, Lys658, Asp679, and Gly681 are conserved in all Zn\textsuperscript{2+}-ATPases (3). **B** shows the membrane topology of HMA2 based on its sequence. The Strep tag sequence is boxed.

**RESULTS**

Fig. 1A shows the membrane topology of HMA2 based on its homology to other P\textsubscript{1B}-ATPases (10). 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. Fig. 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 HH repeats with three of them arranged in a CCX-HXH pattern. In addition, the sequence DSGCCGXSQQPHQHexQ appears twice. All these distinct sequences might potentially contribute to metal binding sites.

Sequence analysis of Zn\textsuperscript{2+}-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. 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, KKKSC, CCG/DXK, QSCHN/EK, CCRSYSYAK, and CSHX\textsubscript{n} (n = 3–11) that can certainly have this role.

To explore the functional role and metal binding characteristics of HMA2 C-MBD, various protein constructs were designed (Fig. 2A). The C-MBD 244 amino acid fragment of HMA2 was expressed in a soluble form and affinity purified (Fig. 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 terminal 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 \pm 0.14 and \(\Delta\)NC-HMA2 = 1.36 \pm 0.13). These differences were later considered in ATPase activity determinations.

**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, A and B). The role of C-MBD appears independent of the presence of the N terminus of the enzyme because no significant kinetic differences were detected among \(\Delta\)C-HMA2 and \(\Delta\)NC-HMA2 proteins. Both truncated proteins exhibit similar \(V_{\text{max}}\) 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\textsuperscript{2+} or Cd\textsuperscript{2+}. Keeping in mind that \(V_{\text{max}}\) is measured at saturating metal concentrations, it is clear that the small changes in activating metal affinity do not explain the reduction of \(V_{\text{max}}\). It is also important to point out that the removal of HMA2 C-MBD had no effect on the relative activation by Zn\textsuperscript{2+} and Cd\textsuperscript{2+} or the relative enzyme affinity for each of these metals; i.e. all three proteins had 4–6 times higher affinity for Cd\textsuperscript{2+} than for Zn\textsuperscript{2+}. This observation contributes to the idea that the removal of C-MBD affects enzyme velocity without changing metal binding to transmembrane transport sites.

**Metal Binding to C-MBD**—Table 1 shows the determination of metal binding to C-MBD by Atomic Absorption Spectroscopy (AAS). This indicated that C-MBD indeed binds Zn\textsuperscript{2+} and Cd\textsuperscript{2+} with a stoichiometry of three metals per C-MBD molecule. It is interesting that the stoichiometry of Zn\textsuperscript{2+} binding is unchanged under reducing (in the presence of TCEP) or non-reducing conditions. On the contrary, binding of Co\textsuperscript{2+} to the C-MBD fragment was affected by the presence of TCEP suggesting a different binding site for the nonactivating metals.

**Zn\textsuperscript{2+} Titrations of C-MBD**—Because Zn\textsuperscript{2+} is spectroscopically silent, to determine the affinity of C-MBD for Zn\textsuperscript{2+}, we...
performed a competition assay with the fluorescent Zn²⁺ indicator mag-fura-2 (45). Mag-fura-2 forms a 1:1 complex with Zn²⁺ with a $K_a$ of $5 \times 10^7$ M⁻¹ (45). 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 $\varepsilon_{366} = 1880$ M⁻¹ cm⁻¹; mag-fura-2-Zn $\varepsilon_{366} = 29900$ M⁻¹ cm⁻¹) (44). Fig. 4A shows the changes in mag-fura-2 spectra upon binding increasing Zn²⁺ levels in the presence of C-MBD. Fitting of mag-fura-2 A$_{366}$ versus free [Zn²⁺] allowed us to calculate the affinity of C-MBD for Zn²⁺ ($K_a = 1/K_a = 15.6 \pm 2.6$ nM) and the apparent stoichiometry of the interaction (2.97 ± 0.13).
A. thaliana HMA2 C-MBD

**FIGURE 4.** Zn

\( ^{2+} \) binding to C-MBD. A, representative spectra of titration of 10 \( \mu \)M C-MBD and 20 \( \mu \)M mag-fura-2 with increasing Zn

\( ^{2+} \) concentrations (5–100 \( \mu \)M). The arrow shows the direction of absorbance change at 366 nm as increasing concentrations of Zn

\( ^{2+} \) are added. B, determination of \( K_a \) for Zn

\( ^{2+} \) binding, and the number of metal binding sites in C-MBD. The data were fit to

\[ \nu = n K_a [Zn^{2+}] / (1 + K_a [Zn^{2+}]), \]

with \( n = 2.97 \pm 0.13 \) and \( K_a = 6.4 \pm 0.9 \times 10^7 \) \( M^{-1} \). Values are the mean \( \pm \) S.E. (\( n = 3 \)).

Zn

\( ^{2+} \)/C-MBD) (Fig. 4B). This last parameter correlates with the determination of metal bound to C-MBD by atomic absorption spectroscopy under saturating metal conditions (Table 1). On the other hand, the observed \( K_a \) value is similar to that described for other Zn

\( ^{2+} \)-binding proteins (38, 44, 46).

**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 function requires characterization of its overall structure and description of the metal binding sites. Fig. 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. 5). 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 1).

**FIGURE 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.

Zn K-edge XAS of Zn

\( ^{2+} \)-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 an average of the three sites. The Zn K-edge XANES spectra (Fig. 6) for C-MBD with one Zn atom per peptide, one Zn atom per Cys-alkylated peptide, and three Zn atoms per peptide, all show a Zn edge that has an energy appropriate for Zn

\( ^{2+} \) centers (energy at a normalized intensity of 0.5 = 9663.7 eV), with no pre-edge transitions, as is typical for Zn

\( ^{2+} \) 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

\( ^{2+} \) complexes (53). 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. 6).

EXAFS analysis for C-MBD peptide containing one Zn

\( ^{2+} \) ion (Fig. 6) is consistent with an average Zn site composed of a N(O),S ligand donor atom set (Table 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 supplementary data). 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 5-coordinate species with either one or two sulfur donors had slightly better values of g.o.f. (N,S g.o.f. = 0.48, N,S,S g.o.f. = 0.50); however somewhat larger values for \( \sigma^2 \) and an increase in the \( \Delta E_0 \) for the S donor(s) (see supplementary data). 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 coordi-
EXAFS analysis for the complex of the Cys-alkylated C-MBD peptide with one Zn$^{2+}$ ion is consistent with an (N/O)$_4$ (Ni-N/O = 1.98 (1) Å) ligand donor atom set with 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. 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.

EXAFS analysis for the Zn C-MBD complex with three Zn$^{2+}$ ions (Fig. 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)$_3$S ligand donor set or a (N/O)$_4$ donor set (Table 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 2–3imidazoles in the coordination sphere regardless of whether a S donor 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$^{2+}$ 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)$_4$ and (N/O)$_3$S coordination. In fact, if non-integer values are used in the fit, an improved fit (g.o.f. = 0.73) (Table 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 ligands, and a third site that is similar to that seen in the sample with one Zn$^{2+}$ 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 supplementary data).
A. thaliana HMA2 C-MBD

**TABLE 2**

| Sample       | N(donor) | R      | \( \sigma^2 \) | \( \Delta E_0 \) | g.o.f. |
|--------------|----------|--------|----------------|----------------|--------|
| 1Zn1CMBD     | 3 N      | 1.98 (2) | 5 (1)         | 3 (1)           | 0.51   |
| 1S          | 2.22     | 7 (2)   | 10 (4)        |                |        |
| 1Zn: 1 Cys-a | 4 N      | 1.98 (1) | 3 (1)         | 4 (1)           | 0.67   |
| 3Zn1CMBD    | 3 N      | 1.99 (1) | 3 (1)         | 4 (2)           | 0.77   |
| 3Zn1CMBD    | 3 N      | 2.23 (6)| 11 (5)        | 5 (9)           |        |
| 3Zn1CMBD    | 3 N      | 2.00 (1)| 4 (1)         | 5 (1)           | 0.73   |
| 3Zn1CMBD    | 0.3 S    | 2.36 (6)| 3 (4)         | 17 (9)          |        |
| 3Zn1CMBD    | 0.3 S    | 2.00 (1)| 4 (1)         | 5 (1)           | 0.78   |

*a* Refers to Cys-alkylated CMBD.

**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 (100× molar excess of TCEP with respect to C-MBD), the number of free Cys was calculated to be 20.3 ± 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 ± 0.6 free Cys per monomer. This reduction in the number of free Cys had no significant effect in the number of Zn\(^{2+}\) binding sites (Table 1). On the other hand, titration of C-MBD with Zn\(^{2+}\) in the presence of mag-fura-2 under non-reducing conditions showed little change in the \( K_d \) of the C-MBD-Zn\(^{2+}\) complex (17.4 ± 1.8 nM), or in the apparent number of metal binding sites (3.6 ± 0.11) (Fig. 7A). In an alternative approach to test the participation of Cys in Zn\(^{2+}\) coordination, the C-MBD was carboxymethylated by treatment with IAA. Surprisingly, although this yielded 0.6 ± 0.2 free Cys per C-MBD peptide, AAS analysis revealed that the modified C-MBD was still able to bind 2.95 ± 0.24 Zn\(^{2+}\) 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\(^{2+}\) titration in the presence of mag-fura-2 (\( K_d \) = 22.1 ± 2.8 nM, \( n \) = 2.5 ± 0.08) (Fig. 7B).

**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. 8, A and B). These results support the participation of His in Zn\(^{2+}\) coordination during binding by C-MBD.

**DISCUSSION**

The key physiological roles of plant Zn\(^{2+}\)-ATPases (10, 11, 41, 54, 55) likely require 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\(^{2+}\)-ATPases (15, 27, 32, 33, 56). In particular, the relatively long C termini have generated attention because they are uniquely associated to eukaryote Zn\(^{2+}\)-ATPases (8, 40, 41). However, no particular metal binding sites are self-evident in these domains and functional complementation studies have not shown a definitive role for them (40, 41). 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.

**The Functional Role of HMA2 C-MBD**—Analysis of the Zn\(^{2+}\)-dependent ATPase activity of HMA2, ΔC-HMA2, and ΔNC-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\(^{2+}\)-ATPases (33), Cu\(^{2+}\)-ATPases (32) and Zn\(^{2+}\)-ATPases (29). Moreover, it is reminiscent of the regulation by various N-and C-terminal cytoplasmic domains observed in many P\(_2\)-type
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\(^{2+}\)-ATPases, also contains a singular N-MBD where the typical CXXC sequence present in Cu\(^{2+}\)-ATPases is replaced by CCXSE (3). This domain also appears to have a regulatory role since N terminus truncated HMA2 has a 50% reduced turnover.\(^3\). Then, it is interesting that also ΔC-HMA2 and ΔNC-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 ΔC-HMA2 with similar relative affinities. Nevertheless, the C-MBD stoichiometrically 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, because in our experiments the on-rate is diffusionally controlled.

The Structure of HMA2 C-MBD—Analysis of plant Zn\(^{2+}\)-ATPase C termini 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 Cys residues do not. Analysis of Zn\(^{2+}\) bound C-MBD with a 1:1 Zn\(^{2+}\):C-MBD stoichiometry indicates that the average Zn\(^{2+}\) site features a (N/O)$_3$S ligand donor atom set (Table 2). The best fit is obtained when all three N donors are being additionally fit as imidazoles. Zn K-edge XAS analysis of Zn\(^{2+}\)-bound C-MBD with a 3:1 Zn\(^{2+}\):C-MBD also fits well to a site with the three imidazole N and one thiolate ligands (Table 2); however, these data can also be modeled equally well with a (N/O)$_3$ ligand set (Table 2) with three imidazole N ligands. Further refinement showed that a best fit can be obtained when the data are modeled to have different Zn\(^{2+}\) 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\(^{2+}\) 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 (63, 64). In these, two of the coordinating
His are arranged as HXH, whereas the third is distantly located more than 20 residues away. HMA2 C-MBD contains a number of HXH repeats with at least three HX₆HXH (n ≥ 20) arrangements. Therefore, it is tempting to hypothesize that the His in HXH repeats are involved in Zn²⁺ coordination. The model containing two (N/O)₅Zn²⁺ sites and one (N/O)₅S Zn²⁺ site is also consistent with sequence data (Fig. 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 Zn²⁺ 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 (64). Also site-directed mutagensis of the ligand binding site of the Staphylococcus aureus Zn²⁺ 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 (65). This again supports the possible replacement of a liganding thiol with Asp, Glu or Asn, Gln ligand pairs while retaining a native-like tetrahedral coordination geometry (65). This again supports the possible replacement of a liganding thiol with Asp, Glu or Asn, Gln ligand pairs while retaining a native-like tetrahedral coordination geometry (65). This again supports the possible replacement of a liganding thiol with Asp, Glu or Asn, Gln ligand pairs while retaining a native-like tetrahedral coordination geometry (65). This again supports the possible replacement of a liganding thiol with Asp, Glu or Asn, Gln ligand pairs while retaining a native-like tetrahedral coordination geometry (65).

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 the C terminus of HMA2 is a novel regulatory metal binding domain with three similar Zn²⁺ binding sites. All three sites appear to contain three His ligands, with one or more of the sites able to bind with a Cys for the fourth ligand.

Acknowledgments—We thank Dr. C. Robert Matthews (University of Massachusetts Medical School, Worcester, MA) for enabling us to perform circular dichroism analysis of C-MBD. We also thank Don Pellegrino (Worcester Polytechnic Institute, Worcester, MA) for his kind help and assistance with AAS determinations. We acknowledge the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory that is supported by the U. S. Dept. of Energy, Division of Materials Sciences and Division of Chemical Sciences. Beamline X9B at NSLS is supported in part by the National Institutes of Health.

REFERENCES

1. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
2. Axelsen, K. B., and Palmgren, M. G. (1998) J. Mol. Biol. 284, 84–101
3. Argüello, J. M. (2003) J. Membr. Biol. 195, 93–108
4. Bull, P. C., and Cox, D. V. (1994) Trends Genet. 10, 246–252
5. Petris, M. J., Mercer, J. F., Culvenor, J. G., Lockhart, P., Gleeson, P. A., and Camakaris, J. (1996) EMBO J. 15, 6084–6095
6. Rensing, C., Ghosh, M., and Rosen, B. P. (1999) J. Bacteriol. 181, 5891–5897
7. Lutsenko, S., and Petris, M. J. (2003) J. Membr. Biol. 192, 1–12
8. Williams, L. E., and Mills, R. F. (2005) Trends Plant Sci. 10, 491–502
9. Axelsen, K. B., and Palmgren, M. G. (2001) Plant Physiol. 126, 696–706
10. Eren, E., and Argüello, J. M. (2004) Plant Physiol. 136, 3712–3723
11. Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., Camakaris, J., Harper, J. F., and Cobbett, C. S. (2004) Plant Cell 16, 1327–1339
12. Mandal, A. K., Yang, Y., Kertesz, T. M., and Argüello, J. M. (2004) J. Biol. Chem. 279, 54802–54807
13. Arnesano, F., Banci, L., Bertini, I., Ciofi-Baffoni, S., Molteni, E., Huffman, D. L., and O’Halloran, T. V. (2002) Genome Res. 12, 255–271
14. DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W., and Sarkar, B. (1997) J. Biol. Chem. 272, 33279–33282
15. Lutsenko, S., Petruckin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
16. Gitschier, J., Moffat, B., Reilly, D., Wood, W. L., and Fairbrother, W. J. (1998) Nat. Struct. Biol. 5, 47–54
17. Harrison, M. D., Meier, S., and Dameron, C. T. (1999) Biochim. Biophys. Acta 1453, 254–260
18. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
19. Arnesano, F., Banci, L., Bertini, I., Ciofi-Baffoni, S., Finney, L. A., Outten, C. E., and O’Halloran, T. V. (2002) J. Mol. Biol. 323, 883–897
20. Hanza, I., Schaefer, M., Klamp, L. W., and Gtilin, J. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13363–13368
21. Larson, D., Mekios, C., and B., Ross, B., Yang, A. S., and Gilliam, T. C. (1999) J. Biol. Chem. 274, 28497–28504
22. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
23. Axelsen, K. B., and Palmgren, M. G. (1998) J. Mol. Biol. 284, 84–101
24. Millis, R. F., Francini, A., Ferreira da Rocha, P. S. C., Baccarani, P. J., Aylett, M., Krieger, G. C., and Williams, L. E. (2005) FEBS Lett. 579, 783–791
25. Forbes, J. R., Hsi, G., and Cox, D. W. (1999) J. Biol. Chem. 274, 12408–12413
26. Schaefer, M., Hopkins, R. G., Failla, M. L., and Gitlin, J. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13363–13368
27. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 18939–18944
28. Bulaj, G., Kortemme, T., and Goldenberg, D. P. (1998) J. Biol. Chem. 273, 2395–2399
29. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
30. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
31. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
32. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
50. Andrade, M. A., Chacón, P., Merelo, J. J., and Morán, F. (1993) Protein Eng. 6, 383–390
51. Goto, J. J., Zhu, H., Sanchez, R. I., Nersissian, A., Gralla, E. B., and Valentine J. S. (2000) J. Biol. Chem. 275, 1007–1014
52. Padden, K. M. K., J. F., Trafford, K. T., Yap, G. P. A., Rheingold, A. H., Borovik, A. S., and Scarrow, R. C. (2001) Chem. Mat. 13, 4305–4313
53. Clark-Baldwin, K., Tierney, D. L., Govindaswamy, N., Gruff, E. S., Kim, C., Berg, J., Koch, S. A., and Penner-Hahn, J. E. (1998) J. Am. Chem. Soc. 120, 8401–8409
54. Gravot, A., Lieutaud, A., Verret, F., Auroy, P., Vavasseur, A., and Richaud, P. (2004) FEBS Lett. 561, 22–28
55. Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., Vavasseur, A., and Richaud, P. (2004) FEBS Lett. 576, 306–312
56. Lutsenko, S., and Petris, M. J. (2002) J. Membr. Biol. 191, 1–12

57. Baekgaard, L., Fuglsang, A. T., and Palmgren, M. G. (2005) J. Bioenerg. Biomembr. 37, 369–374
58. Cornelius, F., and Mahmoum, Y. A. (2003) News Physiol. Sci. 18, 119–124
59. Rimessi, A., Coletto, L., Pinton, P., Rizzuto, R., Brini, M., and Carafoli, E. (2005) J. Biol. Chem. 280, 37195–37203
60. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
61. Szinsky, M. H., Mandal, A. K., Arguello, J. M., and Rosenzweig, A. C. (2006) J. Biol. Chem. 281, 11161–11166
62. Szinsky, M. H., Agarwal, S., Arguello, J. M., and Rosenzweig. A. C. (2006) Biochemistry 45, 9949–9955
63. Karlin, S., and Zhu, Z. Y. (1997) Proc. Natl. Acad. Sci. 94, 14231–14236
64. Auld, D. S. (2001) BioMetals 14, 271–313
65. Pennela, M. A., Arunkumar, A. I., and Giedroc, D. P. (2006) J. Mol. Biol. 356, 1124–1136