Zinc Binding to the NH₂-terminal Domain of the Wilson Disease Copper-transporting ATPase

IMPLICATIONS FOR IN VIVO METAL ION-MEDIATED REGULATION OF ATPase ACTIVITY*

Received for publication, December 6, 2001, and in revised form, January 18, 2002
Published, JBC Papers in Press, January 31, 2002, DOI 10.1074/jbc.M111649200

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Mutations in the Wilson disease copper transporting, P-type ATPase lead to the accumulation of toxic levels of copper in the liver, brain, and kidney causing extensive tissue damage and eventual death. The NH₂-terminal domain (~70 kDa), which contains six copies of the heavy metal-associated repeat GMT/HCXXC, is also able to bind zinc. We have used circular dichroism (CD) and x-ray absorption spectroscopy (XAS) to characterize zinc binding to the NH₂-terminal metal-binding domain. These studies have revealed that zinc is able to bind to this domain with a stoichiometry of 6:1, and upon binding, induces conformational changes in the NH₂-terminal domain. These conformational changes are completely different from those previously observed for copper binding to the domain and lead to an overall loss of secondary structure in the domain. The XAS spectra indicate that zinc is ligated primarily by nitrogen atoms and therefore has low affinity for the heavy metal-associated repeat where copper has been shown to bind. The differences between zinc and copper binding may serve as the basis for the metal-ion mediated regulation of the ATPase in vivo.

Copper is an essential element for all forms of life. Although it is required by many developmentally important enzymes, excess copper is toxic due to its free radical generating ability. In response to this potential threat, many organisms have evolved elaborate control mechanisms to tightly regulate the availability of free copper ions in vivo. Failure of these systems usually leads to severe biological consequences. In humans, there are two major genetic disorders of copper metabolism, Wilson disease (WND)¹ and Menkes disease (MNK) (1, 2).

Wilson disease is an autosomal recessive disorder characterized by the toxic accumulation of copper in the body, primarily in the liver, kidney, and brain. The gene responsible for the disease is located on chromosome 13 and has been shown to encode a 1411-amino acid, copper-transporting, P-type ATPase (ATP7B) (3, 4). In line with the observed pathology of the disease, the gene is found to be expressed at high levels in the liver and kidney and at lower levels in the lung, placenta, and brain (3). Immunochemical studies have shown that under steady-state conditions the WND ATPase is localized primarily to the trans-Golgi network where it pumps copper from the cytoplasm into the trans-Golgi network lumen (5, 6). The subcellular localization of the ATPase can be altered by the concentration of copper. Under elevated copper concentrations the ATPase undergoes a reversible, copper-mediated translocation from the trans-Golgi network to a cytoplasmic vesicular compartment (5, 6). Recent studies in polarized hepatocytes suggest that the ATPase translocates to the apical canalicular membrane where it would pump copper directly into the bile (7).

In contrast to Wilson disease, Menkes disease is an X-linked disorder, which is characterized by a global deficiency of copper in the body. As in WND, MNK is caused by mutations in a copper-transporting, P-type ATPase (ATP7A) which shares a high degree of homology with the WND ATPase (8–10). As for the WND ATPase, the MNK ATPase has been shown to undergo copper-dependent translocation from the trans-Golgi network to the plasma membrane (11). Both the WND and MNK ATPases are members of a growing superfamily of soft metal-ion transporting ATPases which have been identified in a variety of organisms (12). This superfamily can be further subdivided into those ATPases which transport Cu(I) and Ag(I) and those which transport Zn(II), Cd(II), and Pb(II). These soft metal transporters have the common features of other P-type ATPases (ATP-binding, phosphorylation, and transduction domains) and in addition have a large NH₂-terminal metal-binding domain. In the WND and MNK ATPases this domain contains six copies of the metal binding motif GMT/HCXXC (HMA, Heavy Metal Associated domain), whereas those involved in transporting zinc, cadmium, or mercury have between one and three copies of this motif (13). Previously we and others have shown that the NH₂-terminal domain from the WND protein is able to bind six copper atoms tightly in addition to binding several other metals with varying affinities (14, 15). Moreover, it has also been shown that copper binding to the NH₂-terminal domains of the WND and MNK

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¹ This work was supported by the Canadian Institute of Health Research Grant MOP-1800 (to B. S.) and United States National Science Foundation Grant MCB-9809350 (to L. Q.), Raymond and Bertha Birks Research Grant MCB, and the W. M. Keck Foundation Grant 1000. The Isotope Centre at the University of Minnesota has provided facilities for the studies presented in this paper. The use of animals described in this paper was approved by the Institutional Animal Care and Use Committee of the University of Minnesota. We are grateful to Dr. E. S. Davis for the synchrotron X-ray data from the University of Minnesota Synchrotron Radiation Center. N. Wang and M. A. Z. have performed XAS spectroscopy at the University of Minnesota-a synchrotron radiation center. We thank D. J. Jones and H. S. for helpful discussions and review of the manuscript.

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¹ The abbreviations used are: WND, Wilson disease; MNK, Menkes disease; CD, circular dichroism; DTT, dithiothreitol; EXAFS, extended x-ray absorption fine structure; GST, glutathione S-transferase; TCEP, tricarbonylphosphine; WCBD, NH₂-terminal copper-binding domain of the Wilson disease copper-transporting ATPase (residues 1–649); HMA, heavy metal-associated repeat (GMT/HCXXC); XANES, extended x-ray absorption fine structure; XANES, x-ray absorption near-edge structure; XAS, x-ray absorption spectroscopy.

This paper is available online at http://www.jbc.org

Vol. 277, No. 16, Issue of April 19, pp. 13409–13414, 2002
Printed in U.S.A.
proteins occurs through a cooperative mechanism (14, 16). Functional studies on the WND and MNK proteins have also shown that at least some of the six NH₂-terminal HMA repeats are required for both copper transport activity and for copper-induced protein translocation (17–19).

We have recently performed a detailed structural analysis of copper binding to the WCBD using CD and XAS (20). This study revealed that copper binding to the WCBD induces significant conformational changes in the domain. This observation together with other functional data leads us to postulate that copper-induced conformational changes in the WCBD stimulate the phosphorylation of the ATPase, thereby initiating the copper transport cycle (20). Although we have previously shown that zinc is able to bind to the WCBD in 65Zn(II) blotting experiments (14), information regarding its binding mode and possible conformational changes has not been available. We report here the first detailed structural analysis of the WCBD with various stoichiometries of zinc using CD and XAS. This analysis has revealed that, although zinc is able to bind to the WCBD, its binding mode and the conformational changes it induces are significantly different from those observed from the binding of copper. These differences between copper and zinc binding may have implications for the regulation of the ATPase in vivo.

EXPERIMENTAL PROCEDURES

Expression and Purification—The cDNA encoding the WCBD which was previously expressed using the GST fusion vector pGEX-4T-2 (14) was subcloned into pGEX-6P-2 (Amersham Bioscience) and expressed in Escherichia coli strain BL21(DE3) after induction with isopropyl-1-thio-β-galactopyranosidase. Following lysis by freeze-thawing, thefusion protein was present in both the soluble fraction and in inclusion bodies. Fusion protein which was localized to inclusion bodies was solubilized in 6 M urea, combined with the soluble fraction, refolded, and purified as previously described (14). The refolded protein was analyzed by CD spectroscopy to confirm that the refolding procedure was successful. Protein concentration was determined using the BCA protein assay (Pierce). The identity of the expressed protein was confirmed by several rounds of amino-terminal sequencing. This procedure yielded fusion protein which was >95% pure as assessed by SDS-PAGE.

Removal of the GST Moiety—The GST moiety was removed from the fusion protein by incubation with the PreScission protease (Amersham Bioscience). One unit of PreScission protease per milligram of fusion protein was added to the protein solution and the reaction mixture was incubated at 5 °C for 48 h. Following this incubation, the proteins were rapidly precipitated by addition of 40% thio-mercaptoethanol and incubated at 4 °C, whereas the DTT present was used to ensure the reduction of protein thiols and serve as a competitive ligand for zinc. To avoid the use of exogenous sulphydryl containing compounds, some samples were reconstituted in the presence of TCEP, a non-sulphydryl reducing agent, instead of DTT. The unbound metal and DTT (or TCEP) were removed by extensive dialysis against 25 mM ammonium acetate, pH 7.5 (25 mM Tris HCl, pH 8.0, for CD samples). The protein concentration was again confirmed by the BCA protein assay, and metal content was assessed by neutron activation analysis. All dialysis buffers were sparged extensively with argon before use and dialysis was performed in sealed containers. Typical concentrations of apo-WCBD protein used in the this procedure were between 1 and 5 mg/mL. XAS samples were lyophilized by first flash freezing them using a dry ice/acetone bath and then lyophilizing them for 4 days on a Freeze-mobile 25XL lyophilizer (VirTis).

XAS Data Collection and Analysis—The lyophilized samples of WCBD reconstituted with various amounts of zinc were packed into EXAFS sample cells (20 × 2 × 2 mm) and sealed with Mylar tape. X-ray absorption spectra were collected between 9479 and 10405 eV at beamline 6-ID-C of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory and beamline BioCAT-18 of the Advanced Photo Source (APS) at Argonne National Laboratory. The data were collected in fluorescence mode at 10–20 K with a flat Si (220) double-crystal monochromator and a 13-element Ge detector at NSLS, and at 50–80 K with a Si (111) double-crystal monochromator and ion chamber detector at APS. The monochromators were calibrated using the edge energy of zinc foil at 9659.0 eV.

The treatment of raw EXAFS data to yield χ has been discussed in detail in review articles (21, 22). The SSEQxafs program was used to extract χ from A exp by using a cubic spline function, including preliminary baseline correction and correction of fluorescence data for thickness effects and detector response. General procedures for analysis using the program SSEQxafs have been presented in other papers (23). The refinements reported were on k² χ data and the function minimized was calculated as χ k = \sum_{n} (k n)^2 \sigma^2 n exp(k n)^2 (Eq. 1) where n is the number of atoms in the shell, k = \sqrt{2 E} (E is the energy in eV), and σ² is the Debye-Waller factor (24). The amplitude reduction factor (A) and the shell-specific edge shift (ΔE) are empirical parameters that partially compensate for imperfections in the theoretical baseline and phase functions (25). Phase and amplitude functions were theoretically calculated using a curved-wave formalism (26). A variation of FABM (fine adjustment based on models) was used here in the analysis procedure with theoretical phase and amplitude functions (27). For each shell, two parameters were refined at one time (ν and σ) and ΔE values were set as determined by using the data for the crystallographically characterized model complex, Pr 3N 3Zn 3(S 2,2,3,5,6- Me 3C 6H 4)(1-methylimidazole) 3 generously provided by Professor E. Penner-Hahn. The bond lengths used were 2.35 Å for zinc-sulfur bonds and 2.05 Å for zinc-N bonds. The fitting results indicate the average metal-ligand distances, the type and the number of scatterers and the Debye-Waller factors, which can be used to evaluate the distribution of the metal-ligand bond lengths in each shell. The EXAFS goodness of fit criterion applied here is:

\[ \chi^2 = \sum (N_{id} - N_{exp})^2 / \sigma^2 \]

(Eq. 2)

where \( \nu \) is the number of degrees of freedom calculated as \( \nu = N_{id} - N_{exp} \), \( N_{id} \) is the number of independent data points, and \( N_{exp} \) is the number of variables that are refined. \( N_{id} \) is calculated as \( N_{id} = N_{id} + 2N_{id} \sigma^2 + 2\sigma^2 + 2\sigma^2 \epsilon_\nu \), and \( \epsilon_\nu \) is the estimated uncertainty of the data (usually set at 1) (30). The use of \( \chi^2 \) as the criterion for the goodness of fit allows us to compare fits using different number of variable parameters.

Conformational Analysis of WCBD by CD—Samples for CD analysis containing various stoichiometries of zinc were prepared as described above and analyzed on a Jasco J-720 spectropolarimeter. For analysis of changes in secondary structure, samples were loaded into a 0.1-mm path length cell and spectra recorded from 300 to 190 nm. For analysis of changes in tertiary structure, samples were loaded into a 2-mm path length CD cell and spectra recorded from 400 to 250 nm. The spectra were corrected for the contribution of the buffer noise reduced, and the data were converted to molar ellipticity. Molar ellipticity values per residue were calculated by dividing the calculated molar ellipticity by the number of residues in the WCBD (649 residues). The concentration of the protein solution was 14.5 μM.
RESULTS

CD Spectral Analysis—Reconstitution of the WCBD with zinc indicates that it is able to bind zinc with a stoichiometry of 6:1. Addition of copper to a sample of refolded apo-WCBD results in Cu-WCBD with CD spectra which is very similar to that of soluble WCBD (Fig. 1A). This confirms that the refolding procedure has produced a natively folded WCBD which is able to bind copper. Samples of the WCBD containing 0, 2, 4, or 6 bound zinc atoms were prepared by the addition of zinc to the apo-WCBD as described previously, and CD spectra were obtained. Spectra were collected in both the far and near UV regions to examine changes in secondary and tertiary structure, respectively. Binding of increasing amounts of zinc to the domain gives rise to an overall loss in secondary structure content (Fig. 1B). While the addition of either 2 or 4 zinc atoms results in a relatively small change in the far UV CD spectra, the binding of 6 atoms of zinc induces a sharp decrease in ellipticity. These results indicate that, although zinc is able to bind to the domain fairly tightly, it seems to destabilize the domain relative to the native structure. The CD spectra of the 6:1 complex is still indicative of a folded protein, however; the sharp decrease in ellipticity compared with apo-WCBD suggests that a certain amount of secondary structure content (α-helices and/or β-sheets) has been lost. These results are in sharp contrast to those observed for copper binding to the WCBD which show a progressive increase in secondary structure content as copper is bound by the domain (20).

Changes in the near UV CD spectra, indicative of tertiary structure changes, are also observed as zinc binds to the WCBD (Fig. 1C). The largest changes in line shape and ellipticity occur between 270 and 290 nm, with the largest change in intensity occurring upon the addition of the first four equivalents of zinc. Addition of two more equivalents of zinc to form the 6:1 complex did not produce any substantial changes relative to the 4:1 complex. These changes are relatively small when compared with those observed for copper binding to the WCBD where large changes in line shape and ellipticity are observed at 260, 290, and 330 nm (20). The presence of positive ellipticity at 330 nm is indicative of the presence of disulfide bonds which would be expected in the absence of metal (31). When the WCBD is titrated with copper, ellipticity at 330 nm is lost and falls to zero in the 6:1 complex as copper is bound to the cysteine residues in the HMA domain (20). In contrast, when the WCBD is titrated with zinc some ellipticity at 330 nm is lost but not completely eliminated (Fig. 1C). The presence of disulfide bonds in the 6:1 complex indicates that the bound zinc atoms may not be using cysteines as their primary ligands. The CD results suggest that the mode of zinc binding to the WCBD is substantially different from that observed for copper, which is bound with distorted linear coordination using two cysteine residues of each HMA motif (20).

Analysis of XAS Data—To gain a better understanding of the zinc-binding sites in the WCBD, XAS data were collected on five samples with 2, 4, or 6 bound zinc ions (Table I). Following reconstitution with zinc, all samples were lyophilized to maximize sample stability prior to XAS analysis. In a previous study (20) where a similar analysis was carried out on copper reconstituted WCBD, we observed that both liquid and lyophilized samples gave similar XAS spectra indicating that lyophilization did not have an adverse effect on metal coordination. The 2:1 and 4:1 samples were reconstituted in the presence of DTT substituted with the indicated ratio of zinc as determined using neutron activation analysis and the BCA protein assay. Spectra were recorded as described under “Experimental Procedures.” A, CD spectra of WCBD from soluble fraction, after metal removal and refolding (Apo) and after addition of copper to refolded apo-WCBD. Spectra were normalized for differences in protein concentration. B, far UV CD spectra (secondary structure region). C, near UV CD spectra (tertiary structure region).
or TCEP to determine whether the data would be affected by the addition of exogenous sulfhydryl ligands. All samples exhibit an edge energy of around 9662.8 eV, which is slightly higher than that of the Zn(II) model compounds used. The normalized x-ray absorption near edge structure (XANES) data for the five zinc-WCBD samples are quite similar to each other with two poorly resolved bumps after the edge jump as shown in Fig. 2. Due to the poor resolution, not much information can be obtained regarding the nature of the zinc ligation, although the line shape observed is as broad as model complexes of peptides with N scatterers (32).

The EXAFS spectra of the five zinc-WCBD samples, shown in Fig. 3, generally resemble each other both in \( k \)- and \( r \)-space. All samples exhibit a prominent peak centered at \( r = 1.6 \) Å and a very minor peak at \( r = 2.0 \) Å in the \( r \)-space spectra. Quantitative curve fitting results provide useful metrical information and are summarized in Table I. The major feature observed in all samples cannot be fitted with sulfur scatterers as seen by the large goodness-of-fit values. However, fitting that feature with 3–5 nitrogen scatterers at 2.03(2) Å results in significantly smaller goodness-of-fit values. Such distances are consistent with zinc-imidazole ligation (32).

The much weaker peak at \( r = 2.0 \) Å can be modeled with a very small amount of S scattering at 2.31 Å, typical of zinc thiolate bonds (32). For the 2:1 and 4:1 samples, only a small improvement in the quality of the fits is observed (Table I), suggesting that zinc-sulfur coordination does not make a significant contribution in these samples. However, for the 6:1 DTT sample, the goodness-of-fit value decreases by a factor of two, suggesting that the zinc-sulfur scattering contribution becomes more important in the presence of more than 4 zinc per protein. The amount of the sulfur scatterer included in the fits increases slightly with the zinc-protein ratio, from an average of 0.4 sulfur per zinc the 2:1 and 4:1 complexes to 0.7 sulfur per zinc in the 6:1 sample. Although the latter value may appear small on a per zinc basis, within the context of the entire protein unit this value can correspond to 1 zinc ion among the six bound having four sulfur ligands (or 2 zinc with 2 sulfur ligands), since EXAFS analysis can provide only the average coordination environment of the six metal-binding sites. Thus at lower zinc loadings, at most 1.6 sulfur per protein is involved in metal binding. This value increases to 4 in

| Sample | Fit | Zinc – sulfur/nitrogen | \( n \) | \( R \) (Å) | \( \sigma^2 \) | Goodness of fit \( \chi^2 \times 10^4 \) |
|--------|-----|------------------------|------|------|--------|-------------------|
| 2:1 (DTT) | 1 | S | 1 | 2.300 | 0.0000 | 21.1 |
| 2 | N | 1 | 2.035 | 0.0050 | 7.1 |
| 3 | N | 1 | 2.031 | 0.0003 | 7.6 |
| 4 | N | 1 | 2.023 | 0.0024 | 5.8 |
| | S | 0.5 | 2.009 | 0.0013 | |
| 2:1 (TCEP) | 1 | S | 1 | 2.030 | 0.0003 | 20.7 |
| 2 | N | 5 | 2.039 | 0.0052 | 5.1 |
| 3 | N | 4 | 2.037 | 0.0004 | 5.5 |
| 4 | N | 4 | 2.031 | 0.0030 | 3.9 |
| | S | 0.3 | 2.031 | 0.0007 | |
| 4:1 (TCEP) | 1 | S | 1 | 2.231 | 0.005 | 18 |
| 2 | N | 4 | 2.047 | 0.0047 | 4.9 |
| 3 | N | 4 | 2.039 | 0.0049 | 3.9 |
| | S | 0.3 | 2.318 | 0.0012 | |
| 4:1 (DTT) | 1 | S | 1 | 2.250 | 0.0045 | 18 |
| 2 | N | 5 | 2.052 | 0.0086 | 6.1 |
| 3 | N | 4 | 2.051 | 0.0064 | 7.0 |
| 4 | N | 4 | 2.036 | 0.0059 | 5.1 |
| | S | 0.4 | 2.311 | 0.0006 | |
| 6:1 (DTT) | 1 | S | 1 | 2.251 | 0.007 | 18 |
| 2 | N | 4 | 2.03 | 0.0071 | 8.3 |
| 3 | N | 3 | 2.03 | 0.0030 | 9.9 |
| 4 | N | 4 | 2.02 | 0.0038 | 4.6 |
| | S | 0.6 | 2.32 | 0.0009 | |
| 5 | N | 3 | 2.014 | 0.0016 | 4.2 |
| | S | 0.7 | 2.007 | 0.0013 | |

Fig. 2. Normalized zinc K-edge XANES spectra of zinc-WCBD samples. All spectra were plotted on the same scale and have been offset vertically for clarity. Two arrows indicate the peaks at 9640 and 9670 eV, respectively.
Zinc Binding to the Metal-binding Domain of ATP7B

Fig. 3. Fourier-transformed EXAFS spectra (dotted lines) and fitting (solid lines) of all zinc-WCBD samples.

The detailed structural analysis of zinc binding to the WCBD presented here has increased our understanding of the metal binding properties of this domain and has revealed a possible structure-based mechanism for the discrimination of different metal ions in vivo. The conformational effects of zinc binding to the WCBD are completely different from those observed for the binding of copper. The binding of copper was observed to initiate a series of conformational changes (in both secondary and tertiary structure) which lead to an overall stabilization of the WCBD as evidenced by significant increases in ellipticity in the far UV CD spectra (20). The near UV CD spectra of the copper-titrated WCBD also showed the progressive disappearance of disulfide bonds as copper binds to the conserved cysteines residues in the HMA domains. In contrast, the structural changes induced by zinc binding to the WCBD seem to have a destabilizing effect and the near UV CD spectra indicate that zinc ligation may not involve cysteine residues to the same extent as has been observed for copper. Overall, the CD analysis indicates that zinc binds to the WCBD by a very different mechanism from copper.

XAS analysis of the zinc-reconstituted WCBD samples supports the CD results and indicates that the ligation environment for zinc is very different from that for copper. The ligation environment for copper consists of a distorted linear arrangement of two sulfur ligands (most likely from the HMA domain) with a copper-sulfur bond length of 2.17–2.19 Å (20). This is similar to what is found for the copper-binding sites in the MNK metal-binding domain (33). The best fits of the EXAFS spectra from the zinc-reconstituted samples indicate a ligation environment consisting of 3–5 nitrogen scatterers with a zinc-nitrogen distance of 2.03(2) Å. This bond distance is quite consistent with those found in model zinc-imidazole complexes (32). The XAS results also indicate a relatively minor contribution of sulfur atoms to the zinc coordination environment, indicating a low affinity of zinc for sulfur ligation in this protein environment. This low affinity can probably be ascribed to an incorrect ligation geometry for zinc (see below). Instead we find that the preferred zinc-binding sites in the WCBD are composed of nitrogen ligands (most likely from histidine side chains). Both the XANES and EXAFS spectra for all the reconstituted samples are very similar, indicating that most of the zinc-binding sites have a more or less homogeneous composition. Although zinc and copper appear to bind at different sites, we have shown through competitive 65Zn blotting experiments that zinc is released when zinc reconstituted WCBD is exposed to copper (14). This suggests that the conformational changes induced by copper preclude the binding of zinc and that the converse is not true. Furthermore, in competitive 65Zn blotting experiments, the binding of zinc to the WCBD could not be competed away by a 33-fold excess of either calcium or magnesium, suggesting that zinc is not binding non-specifically to the WCBD (14).

At first glance, the lack of sulfur ligation in the zinc reconstituted samples may be somewhat disconcerting in light of the fact that there are six copies of the conserved HMA domain present at the NH2 terminus of the WCBD. However, this can be readily explained by examining the ligation geometry preferences of the metals in question. It has been shown that copper binding to the metal-binding domains of the MNK and WND ATPases occurs through a distorted linear coordination geometry involving two sulfur ligands (20, 33). This type of ligation has also been observed in a single metal-binding domain (Ag(I) reconstituted) from the MNK ATPase by NMR (34). An examination of zinc binding environments in proteins illustrates that zinc prefers to be ligated by histidine residues or a combination of histidine and cysteine ligands with tetrahedral geometry (35, 36). Well known examples of these are the zinc finger proteins in which the zinc atom is tetrahedrally coordinated by four residues, usually a combination of histidine and cysteine (37). Taking these considerations into account, it is not unreasonable to expect that zinc would not be able to occupy the same linear binding site as copper. The WCBD contains 16 histidine residues and 18 cysteine residues, of which 6 are not conserved. These histidine residues and, to a lesser degree the
cysteine residues are most likely acting as the ligating residues in the zinc reconstituted WCBD.

The HMA domain (usually only 1 copy) is also present at the NH\(_2\) terminus of several bacterial zinc transporting ATPases (12, 38, 39). The NH\(_2\) terminus of ZntA and ZiaA, zinc transporting ATPases from *E. coli* (accession number P37617) and *Synechococcus* sp. (accession number Q59998), respectively, also contain other possible metal-binding domains: ANDC-CCGGACST in ZntA and HKHPHSREGHSHSH in ZiaA (12, 39). However, it seems that at least for ZntA (which can also transport Pb(II) and Cd(II)) the HMA alone is sufficient for binding its substrate metals while the additional cysteine-rich domain serves to increase the affinity of metal binding.\(^2\)

In a previous study, where we investigated copper-induced conformational changes in the WCBD, we postulated that this conformational change would help facilitate the phosphorylation of the ATPase, which would then lead to initiation of the catalytic cycle (20). Extension of this hypothesis to the current results would predict that the binding of zinc would not be able to elicit the same response as the binding of copper would. Recent studies suggest that the NH\(_2\)-terminal domains of soft metal ATPases interact in a metal-specific manner with the rest of the pump (40). Chimeric proteins consisting of the ZntA core domain and the WCBD were not able to transport copper but retained the ability to transport zinc, albeit at a 2-fold slower rate. This chimeric behavior in a similar manner to wild-type ZntA lacking its NH\(_2\)-terminal domain, implying that the WCBD cannot replace the functionality of the ZntA NH\(_2\)-terminal domain. These data suggest that the determinants for metal specificity reside in the ATPase core domain and not in the NH\(_2\)-terminal domain.

A study by Voskoboinik *et al.* (41) which examined acyl-phosphorylation of the MNK copper ATPase in yeast has provided several observations which would support this idea. In this study the authors mutated HMA's 1–3 or 1–6 by changing the conserved cysteines to serine residues and measured the kinetics of copper transport and the formation of the acyl-phosphate intermediate *in vivo*. The authors found that mutating the first three HMA repeats decreased the catalytic rate by 50% while catalytic activity was nearly undetectable when all six repeats were mutated (41). Further analysis of this mutant *in vitro* indicated that its rate of phosphorylation was 2-fold lower than for the wild-type protein and that it was more susceptible to inhibition by orthovanadate than wild-type. Based on this and other data, the authors suggest that the NH\(_2\)-terminal HMA repeats act as "internal sensors" (41). Copper binding to the HMA repeats results in a transition of the MNK ATPase to the E1 form which facilitates formation of the acyl-phosphate intermediate (and is more resistant to orthovanadate inhibition) and hence initiation of the catalytic cycle.

Taking these observations together with those presented in this study, we propose that metal induced conformational changes in the WCBD are the basis for triggering acyl-phosphate formation and hence the regulation of the ATPase *in vivo*. While copper binding to the WCBD would be able to elicit the "correct" conformational changes to facilitate phosphorylation of the ATPase, and possible translocation out of the trans-Golgi membrane, those induced by zinc would not be able to accomplish this goal. This hypothesis is strengthened by a recent study which has shown that copper and not zinc, specifically modulates the phosphorylation of the WND ATPase and that copper-induced phosphorylation requires the presence of the WCBD (42). In this way the NH\(_2\)-terminal domains of the WND and MNK ATPases would be able to sense the concentration of the "metal" ion in vivo.

\(^2\) B. Mitra, unpublished observations.

**Acknowledgments** — We acknowledge Ron Hancock of the Sowecko Reactor Facility at the University of Toronto for assistance with the neutron activation analysis. We also thank Professor James E. Penner-Hahn for providing the EXAFS data for the model compound Friz99(2.5,5-Me6,6'-Cl)H(1)Cl(1) with ligands, A. Z. thanks Dr. Luca Quaroni and Dr. Jan-Uwe Rhode for assistance with XAS data collection at the National Synchrotron Light Source at Brookhaven National Laboratory (NSLS) and at the Advanced Photon Source at Argonne National Laboratory (APS).

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