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

P1-type ATPases, the Wilson disease-associated Cu(I)-transporter, and ZntA from Escherichia coli are soft metal P1-type ATPases with mutually exclusive metal ion substrates. P1-type ATPases have a distinctive amino-terminal domain containing the conserved metal-binding motif GXCGXXC. ZntA has one copy of this motif while ATP7B has six copies. The effect of interchanging the amino-terminal domains of ATP7B and ZntA was investigated. Chimeric proteins were constructed in which either the entire amino-terminal domain of ATP7B or only its sixth metal-binding motif replaced the amino-terminal domain of ZntA. Both chimeras conferred resistance to lead, zinc, and cadmium salts but not to copper salts. The purified chimeras displayed activity with lead, cadmium, zinc, and mercury, which are substrates of ZntA. There was no activity with copper or silver, which are substrates of ATP7B. The chimeras were 2–3-fold less active than ZntA. Thus, the amino-terminal domain of P1-type ATPases cannot alter the metal specificity determined by the transmembrane segment. Also, these results suggest that this domain interacts with the rest of the transporter in a metal-ion-specific manner; the amino-terminal domain of ATP7B cannot replace that of ZntA in restoring full catalytic activity.

P1-type ATPases, a subgroup of P-type ATPases, transport soft metal ions such as copper, silver, zinc, cadmium, lead, and cobalt across biological membranes (1–4). The functions carried out by these pumps include maintenance of homeostasis of essential soft metals like copper, cobalt, iron, and zinc; confinement of resistance to toxic concentrations of lead, cadmium, copper, zinc, and silver; and delivery of specific metal ions to target enzymes. In humans, Menkes and Wilson diseases are caused by mutations in two Cu(I)-transporting ATPases, ATP7A and ATP7B, respectively (5–9). Wilson disease is characterized by the accumulation of toxic concentrations of copper in the liver, kidney, and brain. ATP7B, the Wilson disease-associated protein, is a 1411 amino acid protein that is localized primarily in the trans-Golgi network where it pumps copper from the cytoplasm into the Golgi lumen (10). ZntA is one of two P1-type ATPases from Escherichia coli; it is specific for Pb(II), Cd(II), and Zn(II) and confers resistance to these metal ions in vivo (11–13).

A distinctive feature of P1-type ATPases is a highly polar amino-terminal domain of variable length. This domain contains 1–6 repeats of a conserved metal-binding motif; this motif is 70–100 residues long and usually contains the sequence GXCGXXC. This motif also occurs in metallothioneins, copper chaperone proteins, and the periplasmic mercury-binding protein MerP, among others (14–16). Both ATP7A and ATP7B have six repeats of the GXCGXXC sequence, whereas the smaller bacterial P1-type ATPases generally have one or two repeats. In some P1-type ATPases, the cysteine-rich amino terminus is replaced by a highly histidine-rich sequence. The isolated amino-terminal domain of ZntA is able to bind different soft metal ions.1 The amino-terminal domain of ATP7B (WND-Cu(1–6)),2 comprising residues 1–649, has been shown to bind six atoms of copper (17–18). Detailed structural analysis of copper binding to this domain showed that each binding site ligates copper in a +1 oxidation state using two cysteine side chains with distorted linear geometry (19). Analysis of copper-induced conformational changes in the amino-terminal domain revealed that both secondary and tertiary structural changes take place upon copper binding. The conformational changes correlate very well with the cooperativity of copper binding to this domain reported earlier (18). These observations together with other functional data led us to postulate that copper-induced conformational changes in WND-Cu(1–6) stimulate the phosphorylation of the ATPase, thereby initiating the copper transport cycle (19).

P1-type ATPases display a high degree of specificity for the

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1 B. Mitra, unpublished observation.
2 The abbreviations used are: WND-Cu(1–6), the amino-terminal domain of ATP7B with all six of its metal-binding motifs; ΔN-ZntA, a mutant of ZntA with residues 2–106 deleted; WND-Cu(1–6)-ZntA, a chimeric protein in which the first 105 residues of ZntA were deleted and replaced by residues 1–650 of ATP7B together with an extra two residues, GT, at the junction; WND-Cu(6), the sixth metal-binding motif of ATP7B; WND-Cu(6)-ZntA, a chimeric protein in which residues 2–105 of ZntA were deleted and replaced by residues 544–650 of ATP7B together with an extra two residues, GT, at the junction; PAGE, polyacrylamide gel electrophoresis.

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ATP7B together with an extra two residues, GT, at the junction; PAGE, polyacrylamide gel electrophoresis.

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metal ions that are transported. For example, ATP7A and ATP7B transport the monovalent cation Cu(I) and possibly Ag(I). On the other hand, ZntA has been shown to be specific for Pb(II), Zn(II), Cd(II), and Hg(II) (20). The determinants of metal ion recognition and specificity for P1-type ATPases are not known. Because there is a high level of sequence similarity among all P1-type ATPases, the basis of metal ion specificity remains an intriguing question. We recently characterized a mutant of ZntA lacking its amino-terminal domain, ΔN-ZntA (21). ΔN-ZntA is fully capable of ATP-dependent soft metal transport and has the same metal specificity as the intact protein, although its activity is slightly lower than that of ZntA. Thus, the core transport domain of ZntA is necessary and sufficient for the recognition and transport of specific metal ions.

In the present work, our goal was to investigate whether the amino-terminal domain of P1-type ATPases also determines specificity toward particular metal ions. Toward this end, we constructed chimeric proteins in which the amino-terminal domain of ZntA was replaced by the amino-terminal domain of the Wilson Cu(I)-transporter ATP7B (Fig. 1). In one chimeric protein designated WND-Cu(1–6)-ZntA, the entire amino-terminal domain of ATP7B, containing all six metal-binding motifs, was attached to ZntA lacking its amino-terminal domain (ΔN-ZntA). In the second chimeric protein, WND-Cu(6)-ZntA, only the sixth metal-binding motif of ATP7B was attached to ΔN-ZntA. It has been reported that the sixth metal-binding domain is necessary and sufficient for the transport activity of ATP7B (22). Both chimeric proteins were able to confer resistance toward Pb(II), Cd(II), and Zn(II) to a zntA-disrupted E. coli strain but not to copper in an E. coli strain disrupted in copA, which encodes a Cu(I)-transporting ATPase (23). Thus, in vivo the chimeras appear to behave in a manner similar to ZntA and ΔN-ZntA but not to CopA. The purified chimeric proteins were active; the ATPase activity was stimulated by stimulation by ATP7B cannot replace that of ZntA in restoring full catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibodies against the hexahistidyl tag were from CLONTECH, Palo Alto, CA. L-α-Phosphatidylcholine (asolecin) was purified prior to use (24). All other chemicals were of the highest commercial grade.

**Construction of WND-Cu(6)-ZntA and WND-Cu(1–6)-ZntA**—The chimeric proteins WND-Cu(6)-ZntA and WND-Cu(1–6)-ZntA were constructed as follows. For WND-Cu(6)-ZntA, a fragment of DNA encoding amino acids 543–666 of ATP7B that contains the sixth copper-binding domain was generated by polymerase chain reaction. An NcoI site was generated at the 5′-end to provide an initiation codon, and a KpnI site was generated at the 3′-end of the fragment using the oligonucleotides 5′-GACCATGGCTAGTCTTACGAGAC-3′ and 5′-CTGTGACCCCGTTATTTCCATTG-3′ together with the plasmid WCBD-pGEX-6P-2 as template. Plasmid WCBD-pGEX-6P-2 was generated by cloning the cDNA encoding the six Cu(I)-binding domains of ATP7B in pGEX-6P-2 (Amersham Pharmacia Biotech) (18). A fragment of DNA encoding amino acids 106–732 of ZntA containing a KpnI site at the 5′-end and an EcoRI site at the 3′-end was generated using the oligonucleotides 5′-GGTTACCAAAAGCCGGCTATTCCCTG-3′ and 5′-CTCGAAATTCTCCTCGAGCAACATT-3′ and the plasmid pZntA as a template (20). The two individual polymerase chain reaction products were subcloned into pGEM-T (Promega). The WND-Cu(6) fragment was excised by restriction digestion using NcoI and KpnI, while the ZntA fragment was excised using KpnI and EcoRI. The two fragments were purified and ligated together with pBAD/MycHis-C (Invitrogen), which was previously digested with NcoI and EcoRI to generate plasmid pWND-Cu(6)-ZntA in which the WND-Cu(6)-ZntA chimera is in-frame with a hexahistidyl tag at the carboxyl terminus. As a first step toward construction of the DNA fragment encoding the chimera WND-Cu(1–6)-ZntA, the existing EcoRI and NcoI sites, located between nucleotides 1325 and 1336 of the coding sequence of WCBD(1–6)-pGEX-6P-2, were disabled by site-directed mutagenesis by using the U.S.E. Mutagenesis Kit (Amersham Pharmacia Biotech). The oligonucleotide used was 5′-GGTTACCAAAAGCCGGCTATTCCCTG-3′ and 5′-CTCGAAATTCTCCTCGAGCAACATT-3′ in which the altered bases, indicated in bold and italics, replaced GGA in the wild-type DNA. This mutation does not change the amino acid sequence of the amino-terminal domain of ATP7B. The resulting altered plasmid was then used as the template in a polymerase chain reaction to generate a fragment of DNA encoding amino acids 1–666 of ATP7B with NcoI and KpnI sites at the 5′- and 3′-ends respectively, by using the oligonucleotide 5′-GGTTACCAAAAGCCGGCTATTCCCTG-3′ and 5′-CTCGAAATTCTCCTCGAGCAACATT-3′ and the same reverse primer that was used to generate WND-Cu(6). The subsequent steps in creating the plasmid pWND-Cu(1–6)-ZntA were essentially the same as described above for the WND-Cu(6)-ZntA chimera.

To ensure that the polyhistidyl group at the carboxyl terminus can be removed when required, the plasmids carrying the chimeras were further modified. A synthetic oligonucleotide with coding sequence for the cleavage site of PreScission protease (human rhinovirus 3C protease) was introduced by an in-frame insertion between the EcoRI and HindIII sites of both plasmids pWND-Cu(6)-ZntA and pWND-Cu(1–6)-ZntA. This insert also contained an XbaI site to facilitate its identification in the plasmid. The oligonucleotides used were 5′-AATTCTCGAGATTTCTGTTCCAGGGGCCCCTCTACGAGAA-3′ and 5′-AGCTTCGCTAGAAGGCCCCTGGAACAGAACTTCCAGG-3′. The two oligonucleotides

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**Fig. 1.** Schematic representations of WND-Cu(6)-ZntA and WND-Cu(1–6)-ZntA.
were phosphorylated at the 5′-end using the T4 polynucleotide kinase, annealed, then ligated with the plasmids that were precut with EcoRI and HindIII. The presence of the insert in the ligated plasmids was confirmed by XbaI digestion.

The fidelity of the sequences of the chimeras was verified by automated DNA sequencing (DNA Sequencing Facility, Center for Applied Genomics, Hospital for Sick Children, Toronto, Canada).

**Sensitivity to Metal Salts**—The sensitivity of *E. coli* strains LMG194, LMG194(zntA::kan), as well as LMG194(zntA::kan) transformed with either pZntA, pWND-Cu(1-6)-ZntA, or pWND-Cu(6)-ZntA to Pb(II), Zn(II), or Cd(II) salts was measured using a basal salts medium from which zinc salts were omitted (20, 25). As a control, LMG194(zntA::kan) transformed with pΔN-ZntA was also tested (21). Cells were grown overnight and then diluted 50-fold in the same medium containing different concentrations of lead acetate, zinc acetate, or cadmium chloride. Cell growth at 37 °C was monitored by measuring the absorbance at 600 nm either after 24 h or at fixed time intervals. The sensitivity of LMG194, LMG194/copA::kan, as well as LMG194/copA::kan transformed with either pCopA2, pWND-Cu(1-6)-ZntA, or pWND-Cu(6)-ZntA to cupric chloride was measured in Luria-Bertani medium (23). Cell growth at 37 °C was monitored by measuring the absorbance at 600 nm either at fixed time intervals or after 5–8 h.

**Expression and Purification ofWND-Cu(6)-ZntA and WND-Cu(1-6)-ZntA**—The chimeric proteins were expressed by growth of LMG194(zntA::kan) transformed with the plasmids pWND-Cu(6)-ZntA and pWND-Cu(1-6)-ZntA at 37 °C in Luria-Bertani medium followed by induction with 0.02% L-arabinose as described earlier for ZntA (20). The chimeric proteins were purified using established protocols for ZntA (20, 21). The ability of the chimeric proteins to confer resistance to lead, zinc, and cadmium salts was tested. Fig. 2 shows growth of LMG194, LMG194(zntA::kan), and LMG194(zntA::kan) transformed with plasmid pZntA, pWND-Cu(1-6)-ZntA, or pWND-Cu(6)-ZntA in minimal media containing different concentrations of lead, zinc, and cadmium salts. The chimeric proteins were able to complement the sensitivity of the zntA-disrupted strain to the same extent as ZntA or ΔN-ZntA, which is a mutant of ZntA lacking the amino-terminal domain (11, 20–21). The ability of the chimeric proteins to confer resistance to lead, zinc, and cadmium salts was tested. In assays where thiols were added, the concentration of cysteine required to generate a constant metal ion/thiolate ion ratio was calculated using a pKₐ of 8.33 for cysteine. Protein concentrations were determined using the biuret method with bovine serum albumin as standard. Data were analyzed with Kaleidagraph for the Macintosh (Synergy Software).

**RESULTS**

The Chimeric Proteins Are Able to Confer Resistance to Lead, Zinc, and Cadmium but Not to Copper—We showed earlier that the hypersensitivity of the zntA-disrupted strain LMG194(zntA::kan) to lead, zinc, and cadmium salts can be complemented by transforming it with a plasmid containing the genes for ZntA or ΔN-ZntA, which is a mutant of ZntA lacking the amino-terminal domain (11, 20–21). The ability of the chimeric proteins to confer resistance to lead, zinc, and cadmium salts was tested. Fig. 2 shows growth of LMG194, LMG194(zntA::kan), and LMG194(zntA::kan) transformed with plasmid pZntA, pWND-Cu(1-6)-ZntA, or pWND-Cu(6)-ZntA in minimal media containing different concentrations of lead, zinc, and cadmium salts. The chimeric proteins were able to complement the sensitivity of the zntA-disrupted strain to the same extent as ZntA or ΔN-ZntA. Time courses of growth for the same strains in the absence and presence of 5 μM lead acetate, 50 μM zinc acetate, and 5 μM cadmium chloride were also measured (data not shown). Growth rates for LMG194 as well as for LMG194(zntA::kan) containing pZntA, pWND-Cu(1-6)-ZntA, or pWND-Cu(6)-ZntA were similar, although all four plasmid-bearing strains exhibited an initial lag in growth compared with the wild-type strain LMG194.

ZntA does not confer resistance to copper salts in the copA-disrupted strain, LMG194/copA::kan (23). We tested the ability of the chimeric proteins to confer resistance to copper salts. Fig. 3 shows growth of LMG194, LMG194/copA::kan, and LMG194/copA::kan transformed with plasmids containing CopA, WND-Cu(1-6)-ZntA, or WND-Cu(6)-ZntA in the presence of different concentrations of cupric chloride. Unlike CopA, the chimeric proteins were unable to complement the sensitive phenotype of the copA-disrupted strain.

**Expression and Purification of the Two Chimeric Proteins**—The two chimeric proteins were expressed using the same expression vector used for ZntA. WND-Cu(6)-ZntA was expressed at levels similar to ZntA; the level of expression of WND-Cu(1-6)-ZntA was lower than that of ZntA under similar growth and induction conditions. The WND-Cu(6)-ZntA chimera could be purified to near homogeneity; however, the WND-Cu(1-6)-ZntA chimera appeared to be partially degraded into smaller fragments in vivo. The proteins were purified using Ni(II) affinity chromatography; SDS-PAGE analysis of...
the chimeras is shown in Fig. 4. The molecular masses of WND-Cu(6)-ZntA and WND-Cu(1–6)-ZntA, including the histidyl and Myc tags, are 82 and 139.6 kDa, respectively. It is clear from Fig. 4 that WND-Cu(6)-ZntA could be purified to near homogeneity, but the purified WND-Cu(1–6)-ZntA had smaller degradation products. The size of the smallest fragment corresponded to the size of ΔN-ZntA.

Soft Metal Cation-dependent ATPase Activity of the Chimeric Proteins—Both chimeric proteins were active and displayed ATPase activities that were stimulated by soft metal ions. As previously observed for ZntA and for ΔN-ZntA, pretreatment of the chimeras with dithiothreitol as well as with phospholipids in the assay buffer was required for the ATPase activity. Additionally, the thiolate form of cysteine in the assay buffer increased the activity of both chimeric proteins, as has also been observed with ZntA and ΔN-ZntA (20–21). The soft metal ion-stimulated ATPase activity was measured as a function of the MgATP concentration in the presence of 100 μM Pb(II) (data not shown). The $K_m$ values for MgATP at pH 7.0 and 37 °C for WND-Cu(1–6)-ZntA and WND-Cu(6)-ZntA were 89 ± 9 and 160 ± 25 μM, respectively; the $K_m$ for MgATP for ZntA was 106 ± 13 μM.

Table I summarizes the kinetic parameters of both chimeric proteins together with those of ZntA and ΔN-ZntA for the soft metal-stimulated ATPase activity in the presence of excess MgATP (5 mM each Mg(II) and ATP) at pH 7.0 and 37 °C. For all four proteins, Pb(II) was the best metal ion substrate. In the absence of thiocyanate, the $V_{max}$ of the Pb(II)-stimulated activity for both chimeric proteins was 2-fold lower compared with the value for ZntA. The $K_m$ values for Pb(II)-stimulated activity of WND-Cu(6)-ZntA were similar to those of ZntA, whereas those of WND-Cu(1–6)-ZntA were 2-fold lower. The $K_m$ values for all three metals were similar; these $K_m$ values in the absence of thiocyanate refer to the ATP complexes of soft metal ions given the magnitude of the association constants of PbATP, ZnATP, CdATP, and MgATP (29). When thiocyanate were present in the assay buffer at a soft metal ion:thiolate ratio of 1:1, both the $V_{max}$ and the apparent $K_m$ values were higher for all four proteins. The $V_{max}$ values for both chimeras were 2–3-fold lower than that of ZntA; the apparent $K_m$ values were also lower than that of ZntA. These $V_{max}$ and $K_m$ values for the chimeras are very similar to those obtained for ΔN-ZntA (Table I).

The Metal Ion Specificity for the Chimeric Proteins Is Identical to ZntA—Metal cations other than Cd(II), Pb(II), and Zn(II) were tested for their ability to stimulate the ATPase activity of both WND-Cu(6)-ZntA and WND-Cu(1–6)-ZntA. Cu(II), Ni(II), Cu(II), Fe(II), Cr(III), and Bi(III) were unable to stimulate the ATPase activity above background levels. As previously observed for ZntA, Hg(II) was able to stimulate the ATPase activity of the chimeric proteins in the presence of added thiocyanate (20); however, it was less effective than Pb(II), Zn(II), or Cd(II). In the absence of thiocyanate in the assay medium, Hg(II) displayed no activity with ZntA, ΔN-ZntA, or either chimeric protein. Of the divalent metals Co(II), Ni(II), Fe(II), and Cu(II), only Cu(II) inhibited the ATPase activity of the chimeras, as was also true for ZntA and ΔN-ZntA. 5 μM Cu(II) completely inhibited the Pb(II)-stimulated ATPase activity of all four proteins. Because Cu(I) and Ag(I) are substrates of ATP7B, we tested the effect of these monovalent metals on the ATPase activity of the chimeras. Cu(I) (generated from Cu(II) in the presence of excess dithiothreitol) and Ag(I) did not increase the activity of the chimeras above background levels. When testing Ag(I), care was taken to ensure that the lack of Ag(I) activity was not due to its insolubility in the assay buffer.

DISCUSSION

Members of the P1-type ATPase subfamily have a high degree of homology with each other that extends to the hydrophilic metal ion-binding amino-terminal domain, the transmembrane domain, and the ATPase domain. Despite this high level of sequence similarity, P1-type ATPases display stringent specificity in the transport of specific metal ions. For example, ZntA is specific for the ions Pb(II), Zn(II), Cd(II), and Hg(II), while ATP7A, ATP7B, and CopA from E. coli and homologues transport Cu(I) and possibly Ag(I) (5–9, 11–13, 23). Other P1-type ATPases with different metal ion specificities include SizP,
specific for Ag(I), and CoaT, specific for Co(II) (30–31). It is also likely that plants have multiple P1-type ATPases that transport different metal ions including Zn(II), Cu(I), Ni(II), and Mn(II) (32). The molecular determinants of metal ion recognition and specificity in P1-type ATPases are not known. We have previously shown that ΔN-ZntA, a truncated mutant of ZntA that lacks the amino-terminal domain, is an active ATPase with the same metal ion specificity as ZntA, demonstrating that the amino-terminal domain is not essential for function and that the transmembrane domain determines specificity (21). However, ΔN-ZntA was less active than the full-length ZntA, suggesting that the amino-terminal domain may have a function in enhancing the overall catalytic activity, possibly by increasing the rate of metal ion binding to the pump. Studies on the isolated amino-terminal domains of a few P1-type ATPases, notably ATP7A and ATP7B, have shown that these domains can bind a variety of soft metals, including those that are not substrates of the full-length pump (17–19). For example, the amino-terminal domain of ATP7B is able to bind Cu(I), a substrate, as well as Zn(II), Cd(II), and Hg(II), which are ions that are not substrates. Zinc binds to this domain with a stoichiometry of 6:1 and upon binding induces conformational changes that are completely different from those previously observed for copper. Spectra from x-ray absorption spectroscopy indicate that zinc is ligated primarily to nitrogen atoms, which is different from the behavior of copper-binding ligands of this domain. We therefore wanted to investigate the impact of interchanging the amino-terminal domains of P1-type ATPases with mutually exclusive metal ion substrates. In particular, we wanted to determine whether such chimeric proteins are active ATPases and if so, whether the amino-terminal domain is able to alter the specificity of the transmembrane domain. In this study, we have characterized the properties of chimeric proteins constructed by splicing the amino-terminal domains of the mammalian transporter ATP7B with the bacterial transporter ZntA lacking its amino-terminal domain. In one chimera the entire amino-terminal domain of ATP7B, including all six metal-binding motifs, was used; in the second, only the sixth metal-binding domain of ATP7B was used. The sixth metal-binding domain of ATP7B approximately corresponds in size to the amino-terminal domain of ZntA. Also, this domain has been shown to be sufficient for the in vivo transport function of ATP7B in yeast (22).

Both chimeric proteins were able to mediate resistance to lead, zinc, and cadmium salts in a zntA-disrupted strain but not to copper salts in a copA-disrupted strain. Thus, physiologically the chimeras behave like ZntA and not like ATP7B and CopA. Therefore, the amino-terminal domain of ATP7B cannot override the metal ion specificity of ZntA. Additionally, these in vivo results suggested that the chimeric proteins are active transporters. This was confirmed by measuring the ATPase activities of the purified chimeras. Both WND-Cu(1–6)-ZntA and WND-Cu(6)-ZntA could be expressed in functional forms in E. coli, although WND-Cu(1–6)-ZntA appeared to be degraded to some extent in vivo. The purified hybrid proteins were able to catalyze the soft metal ion-dependent hydrolysis of ATP. The $K_m$ values of MgATP for WND-Cu(1–6)-ZntA and WND-Cu(6)-ZntA for the Pb(II)-stimulated activity at pH 7.0 were similar to that of ZntA (90–160 $\mu$M). ZntA is specific for the divalent soft metal cations Pb(II), Zn(II), Cd(II), and Hg(II), with Pb(II) displaying the highest activity (20). The chimeric proteins showed the same substrate specificity, with Pb(II) again displaying the highest activity. In particular, no activity was observed with either chimera for Cu(I) or Ag(I).

A comparison of the $V_{\text{max}}$ and $K_m$ parameters for the ATPase activities of the purified chimeras with those for the ATPase activities of ΔN-ZntA and ZntA in the absence of thiocyanates shows that the Pb(II)- and Zn(II)-stimulated activities of the chimeras are similar to those of ZntA and ΔN-ZntA are nearly 2–3-fold lower than those of ZntA. Interestingly, the Cu(II)-stimulated activity of WND-Cu(6)-ZntA is consistently higher than that of ZntA, ΔN-ZntA, or WND-Cu(1–6)-ZntA. We have previously reported that thiocyanates increase the ATPase activity of ZntA and ΔN-ZntA, possibly by increasing the rate of metal ion release from the transporter (20–21). The effect is most pronounced for the Cd(II)–ATPase activity. Thiocyanates were able to increase the activities of both chimeras for all three metal ions. The thiocyanate-stimulated activities of WND-Cu(1–6)-ZntA, WND-Cu(6)-ZntA, and ΔN-ZntA were highly similar to each other and 2–3-fold lower than those for ZntA (Table I). The thiocyanate-stimulated activity is likely to be a more accurate reflection of the in vivo activity of ZntA, given that glutathione is abundant inside the cell and that metal ion chaperones present in the periplasm may assume the role of thiocyanates. Thus, the data in Table I strongly suggest that the chimeric proteins resemble ΔN-ZntA in terms of their catalytic abilities. Therefore, the amino-terminal domain of ATP7B cannot replace that of ZntA in restoring full catalytic activity.

In contrast to this expectation, Cu(II) in the presence and absence of dithiothreitol did not increase the Pb(II)-stimulated ATPase activity; it completely inhibited the Pb(II)-ATPase activity. This observation suggests

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**Table I**

| Metal ion | ZntA | ΔN-ZntA$^a$ | WND-Cu(1–6)-ZntA | WND-Cu(6)-ZntA |
|-----------|------|-------------|------------------|----------------|
|           | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}$ | $K_m$ |
| Pb(II)    | 502 ± 10 | 5.9 ± 0.4 | 319 ± 6 | 11.6 ± 0.8 |
| Zn(II)    | 186 ± 7  | 5.2 ± 0.7 | 81 ± 7  | 9.3 ± 1.2  |
| Cd(II)    | 83 ± 5   | 3.8 ± 0.8 | 68 ± 3  | 4.1 ± 0.6  |
| Pb(II) + [thiolate] | 2390 ± 201 | 67 ± 4 | 1060 ± 67 | 55 ± 11 |
| Zn(II) + [thiolate] | 730 ± 39 | 115 ± 15 | 372 ± 6 | 42 ± 3 |
| Cd(II) + [thiolate] | 981 ± 43 | 216 ± 19 | 388 ± 10 | 52 ± 5 |

$^a$ Values are from Ref. 21.

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3 M. DiDonato, J. Zhang, L. Que, and B. Sarkar, manuscript in preparation.
that at least in the case of the WND-Cu(1–6)-ZntA chimera the amino-terminal domain does not play a regulatory role.

Conclusion—We constructed two chimeric proteins in which either the entire amino-terminal domain of ATP7B or only the sixth metal-binding domain of ATP7B was attached to ZntA lacking its amino-terminal domain. The chimeras were able to mediate resistance to lead, zinc, and cadmium salts but not to copper salts in vitro. Thus, physiologically, they resemble ZntA. The purified chimeras were active ATPases. The soft metal cation-dependent ATPase activity was specific for Pb(II), Cd(II), Zn(II), and Hg(II), metal ion substrates of purified ZntA; the highest activity was obtained with Pb(II). There was no activity with Cu(I) or Ag(I), which are substrates of ATP7B. The V\textsubscript{max} values for the chimeras are ~3-fold lower than the values for ZntA. Thus, the amino-terminal domain of ATP7B does not override the specificity of the transmembrane segment of ZntA. Also, it cannot replace the amino-terminal domain of ZntA in restoring full catalytic activity to ZntA.

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