The Cysteine-Rich Amino-Terminal Domain of ZntA, a Pb(II)/Zn(II)/Cd(II)-Translocating ATPase from *Escherichia coli*, Is Not Essential for Its Function†

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ABSTRACT: Soft metal-translocating P1-type ATPases have a distinctive amino-terminal domain that contains one to six copies of the conserved metal-binding motif, GXXCXXC. ZntA from *Escherichia coli*, a Pb(II)-, Zn(II)-, and Cd(II)-transporting ATPase, has an ∼120 residue amino-terminal domain with one copy of the GXXCXXC motif as well as four additional cysteine residues. The function of this domain was investigated by constructing a mutant of ZntA lacking the first ∼100 residues. The mutant, ΔN-ZntA, was able to confer resistance to Pb(II), Zn(II), and Cd(II) salts, in a manner similar to ZntA. The soft metal dependent ATP hydrolysis activity of purified ΔN-ZntA was characterized. Purified ΔN-ZntA and ZntA were both inactivated by oxidation. The K_m for MgATP was unchanged for ΔN-ZntA relative to ZntA. ΔN-ZntA displayed the same metal ion specificity as ZntA. Thiolates increased the activities of both ZntA and ΔN-ZntA. The V_{max} values for ΔN-ZntA were ∼3-fold lower than for ZntA for all three metal ions. Thus, the amino-terminal domain is not essential for the function of ZntA or for conferring specificity toward particular soft metals. Its function may be to increase the overall catalytic rate by increasing the rate of metal ion binding to the transporter. Residues involved in the ATP-dependent soft metal ion-translocating mechanism as well as those responsible for recognition of specific metal ions must be part of the core structure of the P1-type ATPases.

P-type ATPases are ATP-dependent pumps that transport cationic substrates across membranes. A distinct evolutionary subgroup of this family, the P1-type ATPases, transports cations such as Cu(I), Ag(I), Zn(II), Cd(II), Pb(II), and Co(II) (*1*−*5*). Physiologically, these pumps maintain homeostasis of the essential metals, Cu(I), Co(II), and Zn(II), as well as mediate resistance to toxic concentrations of Pb(II), Cd(II), Cu(I), and Ag(I); other functions may include delivery of essential cations to target enzymes. P1-type ATPases are widespread in nature; putative members have been found in archea, bacteria, and eukarya. Two human P1-type ATPases, both Cu(I) transporters, have been implicated in Menkes' and Wilson's diseases. ZntA from *Escherichia coli* is a Pb(II)/Cd(II)/Zn(II)-transporting ATPase that mediates resistance to toxic concentrations of Pb(II), Cd(II), and Zn(II) *in vivo* (*6*−*8*). Many ZntA homologues exist in bacteria; eukaryotic ZntA homologues have been identified to date in *Arabidopsis thaliana*.

P1-type ATPases, in common with all P-type ATPases, have a consensus domain for ATP binding and a conserved aspartate residue that is phosphorylated during the catalytic cycle. P1-type ATPases differ from more well-known P-type ATPases, such as the sarcoplasmic reticulum Ca^{2+}-ATPase, in having eight transmembrane helices instead of ten. In addition, P1-type ATPases have a distinctive amino-terminal domain. This highly polar domain contains one to six repeats of a conserved metal-binding motif; this motif is 70−100 residues long and most commonly contains the sequence GXXCXXC. This conserved sequence is also found in metallothioneins, copper chaperone proteins such as Atx1 and CopZ, and MerP, the periplasmic mercury-binding protein, among others (*9*−*11*). The Menkes' and Wilson's disease-associated Cu(I)-ATPases both have six repeats of the GXXCXXC sequence. The isolated amino-terminal domains of both the Menkes and Wilson proteins are able to bind soft metal ions such as Cu(I), Zn(II), Hg(II), and Cd(II) (*12*, *13*). EXAFS studies of these domains indicate that Cu(I) or Ag(I) is bound by two cysteines in a distorted linear geometry (*14*−*16*), a result supported by NMR structural data on the fourth metal-binding motif of the Menkes protein (*17*). While these studies clearly show that the isolated amino-terminal domains of the P1-type ATPases are able to bind soft metal cations, less clear is their role in the ATP hydrolysis and transport activities. Site-specific mutagenesis was used to alter the cysteine pairs in all six of the metal-binding repeats in the Menkes protein expressed in mammalian cells; a transport assay using vesicles showed that the mutant proteins were active, but Cu(I)-induced trafficking was impaired (*18*). However, when similar experiments were carried out with both the Menkes and Wilson proteins expressed in yeast, the results suggested that the amino-terminal domain was essential for activity (*19*, *20*).

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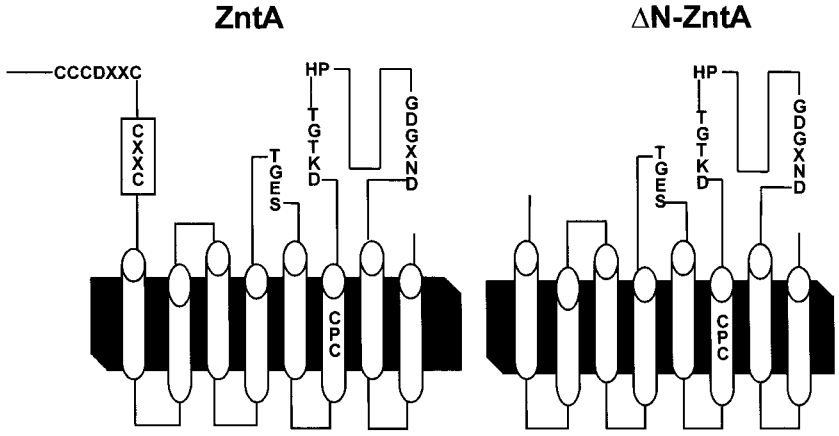


FIGURE 1: Schematic representations of ZntA and ΔN-ZntA.

ZntA from *E. coli* has an ~125 residue long amino-terminal domain that contains a single repeat of the GXX-CXXC motif. In addition, it also contains four additional cysteines, which form part of the sequence CCCDXXC. This latter sequence is not common among P1-type ATPases, though it is found in ZntA homologues from *Salmonella typhimurium* and *Klebsiella pneumoniae*. We recently purified recombinant ZntA and, using the metal ion stimulated ATP hydrolysis assay, showed that it is specific for the metals Pb(II), Cd(II), and Zn(II) (*8*). In the present work, our goal was to investigate the importance of the amino-terminal domain in the function of ZntA. Toward this end, we constructed a truncated version of ZntA that lacks the N-terminal domain and both the CCCDXXC and GXX-CXXC sequences (Figure 1). This protein, designated ΔN-ZntA,[1] is able to confer resistance toward Pb(II), Cd(II), and Zn(II) in a *zntA*-disrupted strain. Purified ΔN-ZntA is able to catalyze soft metal dependent ATP hydrolysis with V_{max} values that are ~3-fold lower than ZntA. Thiolates stimulate the activity of both ZntA and ΔN-ZntA. The specificity displayed by ZntA toward soft metals is unchanged in ΔN-ZntA. The K_ms for Pb(II), Zn(II), and Cd(II) are similar for ΔN-ZntA and ZntA in the absence of thiolates. Our studies clearly demonstrate that the amino-terminal domain is neither essential for the function of ZntA, nor does it play a role in conferring specificity toward particular metal ions. Residues involved in the ATP-dependent cation-translocating mechanism and recognition of specific metal ions must be part of the core structure of P1-type ATPases. The function of the amino-terminal domain may be to increase the overall catalytic rate by increasing the rate of binding of specific metal ions to the transporter.

EXPERIMENTAL PROCEDURES

Materials. E. coli strain LMG194 was obtained from Invitrogen, Carlsbad, CA. L-α-Phosphatidylcholine (asolectin) (Sigma) was purified prior to use (*21*). All other chemicals were of the highest commercial grade.

Methods. Construction of ΔN-ZntA. Plasmid isolation, DNA restriction endonuclease analyses, ligations, and transformations were performed according to established protocol (*22*). The gene for the deletion mutant ΔN-ZntA, with residues 2−106 deleted, was generated by polymerase chain reaction methods using the oligonucleotides 5′ GCGTGC-CATGGCAGGCTATTCC 3′ and 5′ TGAATTCTCTCCT-GCGCAACAATCTTAACG 3′ together with the wild-type *zntA* gene as template. The resulting gene was cloned back into pBAD/*Myc*-His C, the same expression vector as for ZntA, using *Nco*I and *Eco*RI restriction sites to generate plasmid pΔN-ZntA (*8*). The sequence of the mutant gene was confirmed by DNA sequence analysis. As previously described, ZntA was expressed as a carboxy-terminal hexahistidyl-tagged protein under control of the *araBAD* promoter in the *zntA*-disrupted strain, LMG194(*zntA*::*kan*) (*8*). ΔN-ZntA was also expressed as a carboxy-terminal His-tagged protein in LMG194(*zntA*::*kan*).

Sensitivity to Soft Metal Salts. The sensitivity of LMG194 and LMG194(*zntA*::*kan*), as well as LMG194(*zntA*::*kan*) transformed with either pZntA or pΔN-ZntA, to soft metal salts was measured using a basal salt medium from which zinc salts were omitted (*23*). The pH of the medium was adjusted to 7.5. Cells were grown overnight and then diluted 50-fold in the same medium containing different concentrations of lead acetate, zinc chloride, or cadmium chloride. In some experiments, 0.002% L-arabinose was added to all the cultures. Cell growth at 37 °C was monitored by measuring the absorbance at 600 nm either at fixed time intervals or after 24 h.

Expression and Purification of ΔN-ZntA. ΔN-ZntA and ZntA were expressed by growing the pΔN-ZntA or pZntA-transformed LMG194(*zntA*::*kan*) cells at 37 °C in Luria−Bertani medium followed by induction with 0.02% L-arabinose as described earlier (*8*). The purification protocol for ZntA and ΔN-ZntA was as described earlier with the following modification. Prior to extraction of the proteins from the membranes with detergent, the membranes were washed with a buffer containing 2 mM Tris, pH 8.0, 100 mM sucrose, and 1 mM EDTA. This low ionic strength buffer helped to eliminate the F_1 protein from the membranes. This additional step was effective in reducing background ATPase activity that was *not* stimulated by soft metal ions. The purity of the proteins was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

ATPase Assay and Protein Estimation. The soft metal ion dependent ATPase activity was assayed by the pyruvate kinase and lactate dehydrogenase coupled spectrophotometric assay. Purified ZntA or ΔN-ZntA was incubated with 1−2 mM dithiothreitol at 4 °C for 1 h prior to assays. The assay

[1] Abbreviations: ΔN-ZntA, a mutant of ZntA with residues 2−106 deleted; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

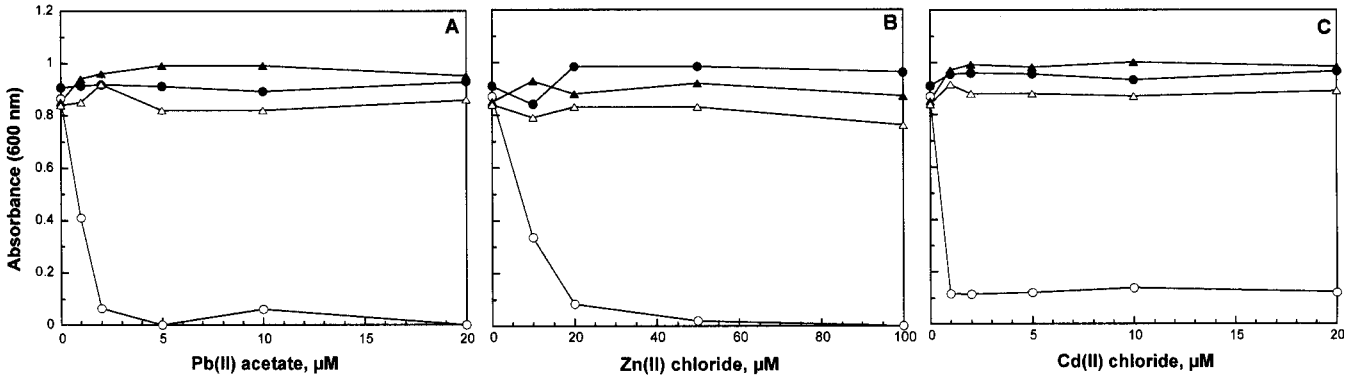


FIGURE 2: Resistance to Pb(II), Zn(II), and Cd(II) salts by the wild-type strain, LMG194 (●), the *zntA*-disrupted strain LMG194(*zntA*::*kan*) (○), and the disrupted strain transformed with the plasmids pZntA (▲) and pΔN-ZntA (△). Cells were grown in a low-phosphate medium in the absence and presence of Pb(II), Zn(II), and Cd(II) salts at 37 °C. Cell growth at the indicated metal salt concentrations was monitored after 24 h by measuring the absorbance at 600 nm. Panels: (A) lead(II) acetate; (B) zinc(II) chloride; (C) cadmium(II) chloride.

buffer was made up of 0.1 M acetic acid, 0.05 M BisTris, and 0.05 M triethanolamine, pH 7.0. The assay mixture also contained 0.1% purified asolectin, 0.1% Triton X-100, 10% glycerol, 5.0 mM each ATP and Mg(II), 0.25 mM NADH, 1.25 mM phosphoenolpyruvate, 14 units of pyruvate kinase, and 20 units of lactate dehydrogenase, with or without soft metal salts. When measuring the K_m for MgATP, the concentration of MgATP was calculated by taking into account the dissociation constants of MgATP and MgHATP and the pK_as of ATP (*24*). Data were fitted to the Michaelis−Menten equation. When the effect of cysteine was investigated, the concentration of the thiolate form of cysteine was calculated using the Henderson−Hasselbalch equation and a pK_a of 8.33. Protein concentrations were determined using the bicinchoninic acid reagent with bovine serum albumin as standard.

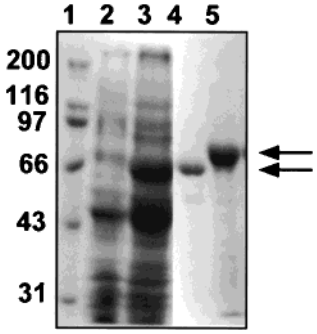


FIGURE 3: Purification of ΔN-ZntA analyzed by 9% SDS−PAGE stained with Coomassie Blue. Lanes: 1, molecular weight markers; 2, membrane fraction of *E. coli* strain LMG194 *zntA::kan* transformed with pΔN-ZntA; 3, Triton X-100 extract from lane 2; 4, purified ΔN-ZntA; 5, purified ZntA. The arrows denote the bands corresponding to ΔN-ZntA and ZntA.

RESULTS

ΔN-ZntA Is Able To Confer Resistance to Pb(II), Zn(II), and Cd(II). The *zntA*-disrupted strain, LMG194(*zntA*::*kan*), is hypersensitive to Pb(II), Zn(II), and Cd(II) salts; this sensitivity can be complemented by transforming it with a plasmid containing ZntA (*7*, *8*). Figure 2 shows growth after 24 h of LMG194 and LMG194(*zntA*::*kan*), as well as LMG194(*zntA*::*kan*) transformed with either plasmid pZntA or plasmid pΔN-ZntA, in minimal media containing different concentrations of Pb(II), Zn(II), and Cd(II) salts. ΔN-ZntA was able to complement the sensitivity of the *zntA*-disrupted strain to the same extent as ZntA. Similar results were obtained when 0.002% arabinose was included in the growth medium. Time courses of growth for the same strains in the absence and presence of 5 μM Pb(II), 50 μM Zn(II), and 5 μM Cd(II) were also measured (data not shown). The rates of growth for both pZntA and pΔN-ZntA-containing LMG194(*zntA*::*kan*) strains were similar in the presence of Pb(II), Zn(II), and Cd(II) salts.

Expression and Purification of ΔN-ZntA. Histidine-tagged ΔN-ZntA was expressed using the same expression vector used for ZntA; the level of expression was slightly better for ΔN-ZntA compared to ZntA under similar growth and induction conditions. As expected, ΔN-ZntA was localized in the membrane fraction of the cells. It could be solubilized from the membranes by a variety of detergents, including Triton X-100, as had been observed earlier for ZntA (*8*). ΔN-ZntA was purified using Ni(II) affinity chromatography; SDS−PAGE revealed that it was >95% pure (Figure 3). The molecular weights of recombinant ZntA and ΔN-ZntA, including the histidyl and *myc* tags, were 80 000 and 69 000, respectively.

Soft Metal Cation Dependent ATPase Activity of ΔN-ZntA. Both ZntA and ΔN-ZntA showed low levels of ATP hydrolysis activities in the absence of Pb(II), Zn(II), or Cd(II) salts. This basal activity is possibly due to trace amounts of contaminating ATPases. However, soft metal ions were able to significantly stimulate the hydrolysis of MgATP for both ZntA and ΔN-ZntA. ATPase activity required the presence of phospholipids in the assay buffer. Similar to our previous observations with ZntA, ΔN-ZntA appeared to lose its activity by oxidation upon storage; treatment with dithiothreitol was required for reactivation. In contrast, the low levels of ATPase activity that were not stimulated by soft metal ions did not require pretreatment with DTT. The 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_ms for MgATP at pH 7.0 and 37 °C for ΔN-ZntA and ZntA were similar, 64 ± 11 and 75 ± 10 μM, respectively.

Table 1 summarizes the kinetic parameters for ZntA as well as ΔN-ZntA for the metal-stimulated ATPase activity in the presence of excess MgATP [5 mM each Mg(II) and ATP] at pH 7.0 and 37 °C (Figure 4). Similar to our previous observation for ZntA, Pb(II) was able to stimulate the

Table 1: Kinetic Parameters Obtained for ZntA and ΔN-ZntA at pH 7.0 and 37 °C for the Metal Ions Pb(II), Zn(II), and Cd(II) in the Absence and Presence of the Thiolate Form of Cysteine Present at a Concentration Equal to the Soft Metal Ion Concentration, [M(II)][a]

	ZntA		ΔN-ZntA	
	V_{max} (nmol·mg^{-1}·min^{-1})	app K_m (μM)	V_{max} (nmol·mg^{-1}·min^{-1})	app K_m (μM)
Pb(II)	584 ± 30	4.8 ± 0.9	319 ± 6	11.6 ± 0.8
Zn(II)	247 ± 13	10.3 ± 1.9	81 ± 7	9.3 ± 1.2
Cd(II)	57 ± 3	5.5 ± 1.2	66 ± 3	4.1 ± 0.6
Pb(II) + [thiolate]	2500 ± 70	166 ± 15	1095 ± 67	55 ± 11
Zn(II) + [thiolate]	710 ± 28	96 ± 11	372 ± 6	42 ± 3
Cd(II) + [thiolate]	1025 ± 25	252 ± 16	388 ± 10	52 ± 5

[a] The Mg(II) and ATP concentrations were 5 mM each. The assay buffer was 0.1 M acetic acid, 0.05 M BisTris, and 0.05 M triethanolamine.

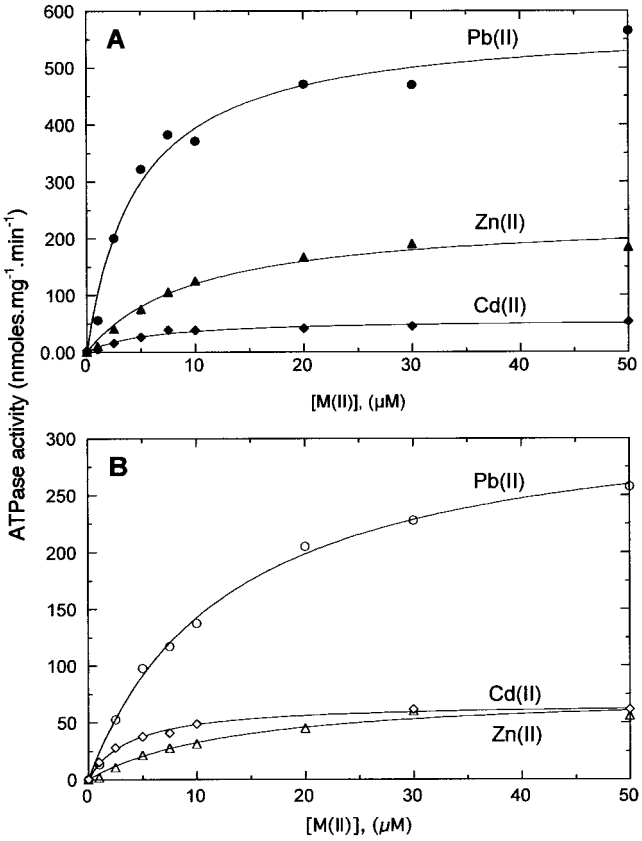


FIGURE 4: ATPase activity of (A) ZntA and (B) ΔN-ZntA as a function of the metal ion concentrations. Assays were carried out at pH 7.0 and 37 °C in the presence of 5 mM each Mg(II) and ATP. Buffer conditions are described in the text. The lines are fits to the Michaelis−Menten equation. Symbols: lead acetate (●); zinc chloride (▲), and cadmium chloride (◆).

ATPase activity of ΔN-ZntA to the highest extent (*8*). The V_{max} for the Pb(II)-stimulated activity for ΔN-ZntA was ~300 nmol·mg^{-1}·min^{-1}, compared to ~580 nmol·mg^{-1}·min^{-1} for ZntA; the K_m for Pb(II) was 2-fold higher for ΔN-ZntA compared to ZntA. The V_{max} for the Zn(II)-stimulated activity was ~3-fold lower for ΔN-ZntA compared to ZntA; the K_ms were similar. Contrary to our previous report that ZntA shows no ATPase activity with Cd(II) (*8*), we observed that Cd(II) also stimulates the ATPase activity of ZntA; however, the activity is much lower than the Pb(II)- or Zn(II)-stimulated activity. The V_{max} and K_m values for the Cd(II)-stimulated activity are similar for ZntA and ΔN-ZntA. The K_m values for the soft metals in the absence of thiolates in Table 1 refer to those of complexes of soft metal ions with ATP since the association constants of PbATP, ZnATP, and CdATP are similar to or higher than that of MgATP (*25*).

Effect of Thiolates on the Soft Metal Ion Dependent ATPase Activity of ZntA. Thiolates of cysteine and glutathione in the assay buffer increase the ATPase and transport activities of ZntA though the apparent K_ms of the metals are much higher in their presence (*8*). We investigated the effect of the thiolate form of cysteine on the Pb(II)-, Zn(II)-, and Cd(II)-stimulated activity of ΔN-ZntA at pH 7.0. Thiolates of cysteine were added at a soft metal ion:thiolate ratio of 1:1. Thiolates were able to increase the activity of ΔN-ZntA. Table 1 summarizes the results obtained with both ZntA and ΔN-ZntA in the presence of thiolates. ΔN-ZntA displayed V_{max} values for all three metal ions that were higher in the presence of thiolates; the V_{max} was ~3.5-fold higher for Pb(II) and Zn(II) and ~5.5-fold higher for Cd(II). In comparison, for ZntA, the V_{max} values were ~4.5-fold higher for Pb(II), ~2.5-fold higher for Zn(II), and ~18-fold higher for Cd(II). As expected, the apparent K_ms for all three metal ions were higher in the presence of thiolates for both ZntA and ΔN-ZntA. When thiolates were added at a metal ion:thiolate ratio of 1:2, essentially similar V_{max} values were obtained as for a metal ion:thiolate ratio of 1:1 (data not shown).

The Metal Ion Specificity for ΔN-ZntA 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 ΔN-ZntA. Co(II), Ni(II), Cr(III), Cu(II), and Fe(II) were unable to stimulate the ATPase activity above background levels. However, Hg(II) was able to stimulate the ATPase activity of ΔN-ZntA in the presence of added thiolates, as was also observed for ZntA (*8*). Since Hg(II) in the absence of added thiolates is a potent inhibitor of the coupled assay system used in this study, the Hg(II)-stimulated activity of ΔN-ZntA was not characterized in detail. It is to be noted that neither ZntA nor ΔN-ZntA confers resistance to Hg(II) in *E. coli*.

DISCUSSION

P1-type ATPases have a distinctive, cysteine-rich metal ion binding amino-terminal domain that is highly conserved; in a few cases it is histidine rich (*26*). The only exception reported so far is CoaT, a Co(II)-ATPase that lacks this amino-terminal domain (*27*). ZntA from *E. coli* has six cysteine residues in its amino-terminal domain, of which two form part of the single GXXCXXC motif. To investigate the role played by this domain in the overall function of ZntA, we constructed a protein lacking this domain. In this study, we characterized both the *in vivo* and *in vitro* properties of this truncated mutant.

ΔN-ZntA Is Able To Mediate Resistance to Pb(II), Zn(II), and Cd(II). The hypersensitivity of the *zntA*-disrupted strain LMG194(*zntA*::*kan*) to Pb(II), Zn(II), and Cd(II) salts could be complemented by plasmids encoding copies of either ZntA or ΔN-ZntA. Time courses of growth in the presence of toxic concentrations of Pb(II), Zn(II), and Cd(II) salts show that strains containing ΔN-ZntA and ZntA have similar growth rates. Therefore, the cysteine-rich amino-terminal domain of ZntA is not essential for mediating resistance *in vivo*.

ΔN-ZntA Shows Metal Ion Stimulated Activity. ΔN-ZntA was expressed in a functional form despite lacking the ~100 residue long, hydrophilic amino-terminal domain. Purified ΔN-ZntA was able to catalyze the metal ion-dependent hydrolysis of ATP. The K_ms for MgATP for ΔN-ZntA and ZntA for the Pb(II)-stimulated activity at pH 7.0 were similar, 65−75 μM. Thus, not surprisingly, the residues involved in the binding of ATP are not part of the amino-terminal domain of ZntA.

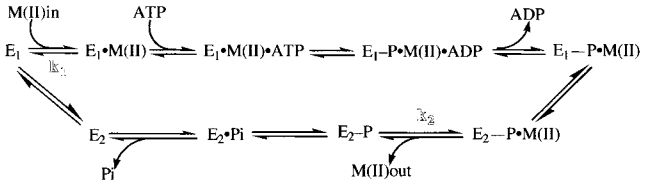
Pretreatment with dithiothreitol was required for both purified ZntA and ΔN-ZntA in order to obtain soft metal ion-stimulated ATPase activity; the low levels of background ATPase activity did not require pretreatment with dithiothreitol. Thus when released from the membrane, both proteins are easily oxidized on exposure to air; the oxidized enzymes are not active. Since ΔN-ZntA does not contain an amino-terminal domain and the GXXCXXC motif, this observation indicates that the conserved CPC motif in the sixth transmembrane domain of ZntA is easily oxidized to cystines in air-saturated buffers. It is to be noted that ΔN-ZntA contains only three cysteine residues in all, including the two in the CPC motif. Thus, the reduced form of the CPC cysteines is essential for activity.

A comparison of the V_{max} and K_m parameters for the Pb-(II)-, Zn(II)-, and Cd(II)-stimulated ATPase activities of purified ΔN-ZntA and ZntA in the absence of thiolates reveals that removal of the amino-terminal domain affects the Pb(II)- and Zn(II)-ATPase activities but not the Cd(II)-ATPase activity. The V_{max}/K_m parameter is ~3−4-fold lower for ΔN-ZntA compared to ZntA for Pb(II) and Zn(II) and essentially similar for the Cd(II)-ATPase activity. Pb(II) is the most efficient substrate for ΔN-ZntA, just as for ZntA. However, while Zn(II) is a much better substrate than Cd(II) for ZntA, this is not the case for ΔN-ZntA.

We have shown earlier that thiolates in the assay buffer increase the ATPase activity of ZntA (*8*). One explanation for this effect is that the metal thiolate complexes are better substrates for ZntA; the cysteines in the amino-terminal domain may be more effective in exchanging the metal cation from thiolate complexes. However, if this were true, then the presence of thiolates in the assay buffer should result in very low or no ATPase activity for ΔN-ZntA, a protein that lacks the amino-terminal cysteines. Contrary to this expectation, thiolates were able to increase the ATPase activities of ΔN-ZntA in a manner similar to that observed with ZntA. The V_{max} values were ~3-fold higher for ΔN-ZntA in the presence of thiolates for all three metal ions, Pb(II), Zn(II), and Cd(II). The increase in activity was similar for both 1:1 and 1:2 metal ion:thiolate ratios. It is to be noted that even in the presence of thiolates the V_{max} values for all three metal ions were lower for ΔN-ZntA than for ZntA. It is clear from these results that thiolates exert their stimulatory effects on the activity by interacting with parts of the transporter other than the amino-terminal domain.

The results presented above agree with site-specific mutagenesis studies of the cysteines in the GXXCXXC motifs in the six metal-binding domains of the Menkes Cu(I) transporter by Voskoboinik and co-workers (*18*). When all six cysteine pairs were altered to serine, the K_m for Cu(I) was essentially unchanged while the V_{max} decreased 4-fold in an *in vitro* transport assay. However, results obtained using yeast complementation assays with site-specific mutagenesis

Scheme 1



of both the Menkes and the Wilson Cu(I) transporters are at variance with our results (*19*, *20*). It is possible that this indirect assay does not accurately report activities of the mutant Cu(I) transporters that are lower than the wild-type values.

Soft Metal Specificity. ZntA is specific for the divalent cations Pb(II), Zn(II), Cd(II), and Hg(II), with Pb(II) displaying the highest activity (*8*). Despite lacking the metal ion binding amino-terminal domain, ΔN-ZntA also showed the same substrate specificity, with Pb(II) again displaying the highest activity. In particular, no activity was observed with either ZntA or ΔN-ZntA for Cu(II), Co(II), or Ni(II). This observation indicates that the determinants of metal ion specificity reside in the core ATPase and transport domains of P1-type ATPases and not in the amino-terminal metal binding domain.

Function of the Amino-Terminal Domain of ZntA in the Overall Activity. The catalytic cycle of P-type ATPases is believed to follow Scheme 1. P1-type ATPases have overall catalytic activities that are much lower than non-soft metal P-type ATPases (*8*, *18*, *28*). One reason for the lower activities may be that the rates of metal ion binding to the E_1 state, k_1, and release from the E_2 state, k_2, for the P1 transporters may be slow processes and rate limiting for the overall reaction. k_1, k_2, or both may be rate-limiting steps depending on the particular metal ion transported. The isolated amino-terminal domains of Menkes and Wilson Cu-(I)-ATPases have been shown to bind many different metal ions, including ones that are not substrates for these pumps (*12*, *13*); this is also true for ZntA (B. Mitra, unpublished observation). These results, together with the comparative study of ZntA and ΔN-ZntA activities presented in this work, suggest that when the amino-terminal domain is present, k_1, the rate of metal ion binding to ZntA, increases. Metal ion binding to the P1-type ATPases may be a one-step or a two-step process. In the single-step process, the amino-terminal domain may contribute residues to an existing cation-binding site on the transporter that makes it a more efficient metal-binding site. In the more likely case of a two-step process, the amino-terminal domain of P1-type ATPases may form an efficient metal-binding site that transfers the cation to a second, less efficient metal-binding site on the transporter. Further experiments are required to distinguish between these possibilities.

Thiolates increase the ATPase activities of both ZntA and ΔN-ZntA. The effect is most pronounced for the Cd(II)-ATPase activity. The thiolate-stimulated activity is likely to be a more accurate reflection of the *in vivo* activities of the pump. This is because the Cd(II) activity of ZntA and ΔN-ZntA in the absence of thiolates is quite low; however, both proteins efficiently mediate resistance to Cd(II) salts. In addition, Cd(II) is the most efficient inducer of ZntA (*29*). One possible explanation for the effect of thiolates is that they increase k_2, the rate of metal ion release from the

transporter (Scheme 1). This effect is expected to be most pronounced for Cd(II), a metal ion that is the most "soft" in character of the three substrates for ZntA, Pb(II), Zn(II), and Cd(II). *In vivo*, metal ion chaperones present in the *E. coli* periplasm may assume the role of thiolates. In the absence of thiolates, ΔN-ZntA has lower activity with both Pb(II) and Zn(II), but not Cd(II), compared to ZntA. This suggests that the rate-limiting step in the absence of thiolates may be metal ion release for Cd(II) transport and metal ion binding for Pb(II) and Zn(II) transport. When thiolates are present, k_1 appears to be rate limiting for all three metal ions.

Conclusion. We have purified and characterized a truncated version of ZntA that lacks the cysteine-rich amino-terminal domain. ΔN-ZntA is able to mediate resistance to Pb(II), Zn(II), and Cd(II) *in vivo*. Purified ΔN-ZntA shows metal ion dependent ATPase activity specifically with Pb(II), Cd(II), Zn(II), and Hg(II) with the highest activity obtained in the presence of Pb(II). The V_{max} values for ΔN-ZntA are ~3-fold lower than the ZntA values. Thus, the amino-terminal domain of ZntA is not essential for its function; it is also not responsible for the metal ion specificity displayed by ZntA. Its function may be to increase the rate of metal ion binding to the transporter and thereby the overall catalytic rate.

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REFERENCES

1. Axelson, K. B., and Palmgren, M. G. (1998) *J. Mol. Evol. 46*, 84−101.
2. Lutsenko, S., and Kaplan, J. H. (1995) *Biochemistry 34*, 15607−15613.
3. Solioz, M., and Vulpe, C. (1996) *Trends Biochem. Sci. 21*, 237−241.
4. Gupta, A., Matsui, K., Lo, J. F., and Silver, S. (1999) *Nat. Med. 5*, 183−188.
5. Gatti, D., Mitra, B., and Rosen, B. P. (2000) *J. Biol. Chem. 275*, 34009−34012.
6. Beard, S. J., Hashim, R., Membrillo-Hernandez, J., Hughes, M. N., and Poole, R. K. (1997) *Mol. Microbiol. 25*, 883−891.
7. Rensing, C., Mitra, B., and Rosen, B. P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14326−14331.
8. Sharma, R., Rensing, C., Rosen, B. P., and Mitra, B. (2000) *J. Biol. Chem. 275*, 3873−3878.
9. Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V., and Culotta, V. C. (1997) *J. Biol. Chem. 272*, 9215−9220.
10. Odermatt, A., and Solioz, M. (1995) *J. Biol. Chem. 270*, 4349−4354.
11. Steele, R. A., and Opella, S. J. (1997) *Biochemistry 36*, 6885−6895.
12. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) *J. Biol. Chem. 272*, 18939−18944.
13. DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W., and Sarkar, B (1997) *J. Biol. Chem. 272*, 33279−33282.
14. Ralle, M., Cooper, M. J., Lutsenko, S., and Blackburn N. J. (1998) *J. Am. Chem. Soc. 120*, 13525−13526.
15. DiDonato, M., Hsu, H.-F., Narindrasorasak, S., Que, L., and Sarkar, B. (2000) *Biochemistry 39*, 1890−1896.
16. Cobine, P. A., George, G. N., Winzor, D. J., Harrison, M. D., Mogahaddas, S., and Dameron, C. T. (2000) *Biochemistry 39*, 6857−6863.
17. Gitschier, J., Moffat, B., Reilly, D., Wood, W. I., and Fairbrother, W. J. (1998) *Nat. Struct. Biol. 5*, 47−54.
18. Voskoboinik, I., Strausak, D., Greenough, M., Brooks, H., Petris, M., Smith, S., Mercer, J., and Camakaris, J. (1999) *J. Biol. Chem. 274*, 22008−22012.
19. Payne, A. S., and Gitlin, J. D. (1998) *J. Biol. Chem. 273*, 3765−3770.
20. Forbes, J. R., Hsi, G., and Cox, D. W. (1999) *J. Biol. Chem. 274*, 12408−12413.
21. Kagawa, Y., and Racker, E. (1971) *J. Biol. Chem. 246*, 5477−5487.
22. Sambrook J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Poole, R. K., Williams, H. D., Downie, J. A., and Gibson, F. (1989) *J. Gen. Microbiol. 135*, 1865−1874.
24. Morrison, J. F. (1979) *Methods Enzymol. 63*, 257−294.
25. Sigel, H. (1987) *Eur. J. Biochem. 165*, 65−72.
26. Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1993) *J. Biol. Chem. 268*, 12775−12779.
27. Rutherford, J. C., Cavet, J. S., and Robinson, N. (1999) *J. Biol. Chem. 274*, 25827-2832.
28. Wunderli-Ye, H., and Solioz, M. (2001) *Biochem. Biophys. Res. Commun. 280*, 713−719.
29. Binet, M. R., and Poole, R. K. (2000) *FEBS Lett. 473*, 67−70.

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