The ATP Hydrolytic Activity of Purified ZntA, a Pb(II)/Cd(II)/Zn(II)-translocating ATPase from Escherichia coli*

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ZntA, a soft metal-translocating P1-type ATPase from Escherichia coli, confers resistance to Pb(II), Cd(II), and Zn(II). ZntA was expressed as a histidyl-tagged protein, solubilized from membranes with Triton X-100, and purified to homogeneity. The soft metal-dependent ATP hydrolysis activity of purified ZntA was characterized. The activity was specific for Pb(II), Cd(II), Zn(II), and Hg(II), with the highest activity obtained when the metals were present as thiolate complexes of cysteine or glutathione. The maximal ATPase activity of ZntA was ~3 μmol/(mg·min) obtained with the Pb(II)-thiolate complex. In the absence of thiolates, Cd(II) inhibits ZntA above pH 6, whereas the Cd(II)-thiolate complexes stimulate activity, suggesting that a metal-thiolate complex is the true substrate in vivo. These results are consistent with the physiological role of ZntA as mediator of resistance to toxic concentrations of the divalent soft metals, Pb(II), Cd(II), and Zn(II), by ATP-dependent efflux. Our results confirm that ZntA is the first Pb(II)-dependent ATPase discovered to date.

P-type ATPases catalyze the ATP-dependent transport of cationic substrates across membranes. These ubiquitous transporters have been classified into five groups according to substrate specificity (1). The rapidly growing and relatively recently discovered ATPases transport cations of metals that are large and easily polarized (termed here “soft metals”) form one of these subgroups, the P1-type ATPases (1–4). Putative soft metal P-type ATPases have been found in archa, bacteria, and eukarya including humans. Their physiological roles appear to be to maintain homeostasis of the essential soft metal, Cu(I), as well as to mediate resistance to toxic concentrations of Pb(II), Cd(II), Cu(I), and Ag(I). The soft metal P-type ATPases can be further subdivided into two classes. One class contains the Cu(I)- and Ag(I)-transporting pumps, which include the human Menkes and Wilson disease-related proteins (5, 6) and the Cu(I)-ATPases from Saccharomyces cerevisiae (7), Enterococcus hirae (8), Synecococcus (9, 10), and Helicobacter pylori (11). The other class of soft metal ATPases have been shown to transport the divalent soft metals, Zn(II) and Cd(II). These include ZntA from Escherichia coli (12, 13) and CadA from Staphylococcus aureus (14). Putative ZntA homologs are widespread in prokaryotes (15), and one has been recently identified in the eukaryote, Arabidopsis thaliana (GenBank accession no. 4210504).

In addition to the many common features of all P-type ATPases, including consensus domains for ATP binding and hydrolysis and a conserved aspartate residue that is phosphorylated during the catalytic cycle, soft metal ATPases have a few distinctive features of their own. One of the most striking features is the presence of 1–6 metal-binding motifs in the polar NH2-terminal segment. This motif is most commonly GMTCXXX, or, in the case of some of the Cu(I) pumps, (M/H)XXMDH(3/4)XXM. In addition, the invariant proline residue found in one transmembrane helix of all P-type ATPases is flanked by two cysteine residues in most of the soft metal pumps; in a few cases, this CPC motif is replaced by CPH, CPS, or SPC. This motif probably plays a role in the translocation of the soft metal cation. Soft metal ATPases also have a conserved histidine-proline dipeptide on the carboxyl-terminal side of the conserved phosphoenzyme-formation sequence.

While ATP-dependent metal transport as well as metal binding by the amino-terminal domains of some soft metal pumps have been shown so far (8, 12–18), metal ion-dependent ATPase activity by these transporters has not been clearly demonstrated. We have initiated biochemical studies of ZntA, as a prototype for the divalent soft metal transporting ATPases. Previously, we showed that ZntA confers resistance to Pb(II), Zn(II), and Cd(II) in E. coli by ATP-dependent efflux of these metal ions (12, 13). In this study, we report the purification of ZntA and the characterization of its soft metal-stimulated ATP hydrolysis activity. ZntA was overexpressed as a hexahistidyl-tagged protein, solubilized from membranes, and purified to homogeneity by affinity chromatography. Purified ZntA displayed ATPase activity that was stimulated specifically by Pb(II) > Cd(II) > Zn(II) > Hg(II), in that order. This suggests that the physiological role of ZntA is to mediate resistance to the toxic metals, Pb(II), Cd(II), and Zn(II). The ATPase activity was highest when the soft metal cations were present as high affinity thiolate complexes. pH dependence studies of the ATPase activity of ZntA indicated that free Cd(II) and Hg(II), or their lower affinity, non-thiolate complexes, inhibit the protein at neutral and alkaline pHs. This suggests that a metal-thiolate complex is the substrate for ZntA in vivo. The maximal ATPase activity of ZntA was observed with Pb(II), with an activity of ~3 μmol/(mg·min) obtained for the thiolate complex of Pb(II) at neutral pH; this value is comparable to those observed previously for Cu(I)- and Na(I)-ATPases (19, 20) and is 2–3 orders of magnitude greater than the values reported for other P1-type ATPases (21, 22). Our data confirm our previous hypothesis that ZntA is a Pb(II)-ATPase (13).

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Experimental Procedures

Materials—Plasmid pBAD/Myc-His C and E. coli strains TOP10 and LMG194 were obtained from Invitrogen, Carlsbad, CA. t-α-Phosphatidylcholine (asolecin) was purchased from Sigma and purified prior to use (23). Monoclonal antibodies against the hexahistidyl tag were obtained from CLONTECH, Palo Alto, CA. Anti-mouse IgG peroxidase conjugate was purchased from Sigma. Zn(II) sulfate was from American Pharmacia Biotech; 10 mM(Cd(II)) acetate was from Los Alamos Laboratories.

Expression of ZntA—ZntA was expressed with a carboxyl-terminal hexahistidyl tag under the tightly controlled araBAD promoter. ZntA was subcloned into the expression vector pBAD/Myc-His C by polymerase chain reaction methods. An NcoI site was generated at the initiation codon in the PCR product, which was subsequently inserted downstream of the termination codon using the oligonucleotides 5′-GATGCCCATGGCAGCTCCTGAGCAATCGGGC-3′ and 5′-TGAAATCTTCTGTCGGCAACATTCTACGGC-3′ together with the plasmid pCGR2, described previously, as template (12). Following restriction digestion with NcoI and EcoRI, pBAD/Myc-His C and the amplified DNA product were ligated together to generate the plasmid pZntA, in which zntA is in-frame with a hexahistidyl tag at the carboxyl terminus, pZntA was transformed into E. coli strains TOP10 or LMG194 as well as the zntA-disrupted LMG194 strain. The zntA disruption was transferred from E. coli strain RW3110, described previously (12), to LMG194 by generalized transduction with P1 bacteriophage, with selection for kanamycin resistance. ZntA was routinely expressed by growing the pZntA-transformed LMG194 (zntA::kan) cells at 37 °C in Luria Bertani medium supplemented with 100 μg/ml ampicillin until the absorbance at 600 nm was 1, followed by induction with 0.02% L-arabinose, unless noted otherwise. Cells were harvested 4 h after induction, washed with ice-cold buffer containing 25 mM Tris, pH 7.0, and 100 mM KCl, and stored at −70 °C till further use.

Transport Assays in Everted Membrane Vesicles—Everted membrane vesicles were prepared from induced LMG194 (zntA::kan) cells with and without pZntA, as described previously (12). Transport assays were performed with ZnSO4 (10 μM) or Cd(II) acetate, usually at pH 6.0. Transport assays were also performed with 20 μM Zn(II) sulfate or 100 mM Cd(II) acetate, usually at pH 7.0.

Subcellular Fractionation of ZntA from Membranes and Purification—All buffers were degassed or sparged with nitrogen before use to minimize the oxidation of the enzyme. Purification steps were carried out at 4 °C. Frozen cells were thawed and resuspended in buffer A (25 mM Tris, pH 7.0, with 100 mM sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF)1) at 0.2 g/ml and disrupted at 20,000 p.s.i. Dounce 1 (0.02 mg/ml) and 2 mM MgCl2 were then added, and the lysed cells were incubated for 30 min. The cells were then diluted 2-fold with buffer A and centrifuged at 229,000 g for 1 h to separate the membranes from the soluble fraction of the cells. The pellet from this centrifugation was washed with buffer A and centrifuged at 229,000 × g for 1 h. The washed membranes were suspended in buffer A and stored frozen till further use.

Frozen membranes were diluted to a protein concentration of 3 mg/ml with buffer B (25 mM Tris, pH 8.0, with 100 mM sucrose, 500 mM NaCl, and 1 mM PMSF). Triton X-100 was added dropwise to a final concentration of 1%, and the suspension was stirred at 4 °C for 1 h under nitrogen. The insoluble fraction was removed by centrifugation at 229,000 × g for 1 h. The Triton X-100 extract (80 ml) was then incubated with 5 ml of Ni(II) resin (Invitrogen, CA), pre-equilibrated with buffer C (buffer B containing 0.5% Triton X-100), and gently mixed for 1 h at 4 °C. The resin was next loaded on a column and washed successively with 25 ml of buffer C, 25 ml of buffer C containing 50 mM imidazole, and 10 ml of buffer C containing 100 mM imidazole. The protein was finally eluted with 10 ml of buffer C containing 300 mM imidazole. 1-ml fractions were collected and analyzed for protein.

Protein-containing fractions were concentrated using Centricon concentrators (Millipore, Bedford, MA), and the imidazole was immediately removed by gel-perfusion on a 20-ml Sephade X G-25 column pre-equilibrated with buffer D (25 mM Tris, pH 8.0, with 100 mM sucrose, 50 mM KCl, 0.2% Triton X-100, and 1 mM PMSF). Fractions were assayed for ATPase activity. The active fractions were pooled (with typical protein concentrations ranging from 0.5 to 1 mg/ml) and stored at −70 °C following quick freezing in liquid nitrogen.

ATPase Activity and Protein Estimation—The metal-dependent ATPase activity of purified ZntA was assayed by the pyruvate kinase and lactate dehydrogenase coupled spectrophotometric assay (24), in which the regeneration of ATP is coupled to the oxidation of NADH. The reaction was monitored at 340 nm. Purified ZntA was rapidly thawed and incubated with 2 mM diethiothreitol at 4 °C for 1 h prior to assays. For the data presented, the enzyme was pre-incubated with 1 mM of the indicated metal chelator for 5 min before initiating the reaction with 5 mM MgCl2. When measuring the Km values of MgATP, the concentration of MgATP was calculated by taking into account the dissociation constants of MgATP, MgHATP, and the pK values of ATP (26). Protein concentrations were determined using the bicinchoninic acid reagent with bovine serum albumin as standard.

In assays where the metal thiolate complex was used, the concentration of the chelator or thiol required to generate the metal:ion-thiolate ion ratio was calculated at each pH using the Henderson-Hasselbalch equation and the pK values of 8.33 and 8.7 for cysteine and glutathione, respectively.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot—Protein expression and purity was assessed by 8–10% SDS-polyacrylamide gels. For Western blotting, the proteins were electrothermally transferred to nitrocellulose membranes following electrophoresis. Monoclonal antibodies against the hexahistidyl tag were used to observe the recombinant protein.

Expression of ZntA—To express ZntA under a heterologous promoter as well as to facilitate its purification, the zntA gene was subcloned with a carboxyl-terminal histidyl tag into the expression vector pBAD/Myc-His C, where it is under control of the tightly regulated araBAD promoter. ZntA was expressed in the E. coli strains Top10 and LMG194 as well as the mutant strain LMG194 (zntA::kan), where the chromosomal copy of zntA was disrupted by insertion of a kanamycin cassette. The recombinant ZntA was able to complement the sensitivity of the disrupted strain to Zn(II) and Cd(II) salts to the same extent as the wild-type strain; complementation was observed only when the inducer, arabinose, was added (data not shown). This demonstrates that expression of recombinant ZntA was tightly regulated and that the protein was functional.

To maximize the expression of ZntA, a variety of growth and induction conditions were examined. For example, both the temperature and duration of growth and induction were varied as well as the inducer (L-arabinose) concentration. Expression was maximal when pZntA-transformed LMG194 (zntA::kan) cells were grown to mid-log phase and then induced with 0.02% arabinose for 4–6 h at 37 °C. Arabinose concentrations lower than 0.02% produced less protein, as did induction for 16 h. There was no detectable expression of ZntA in the absence of arabinose, as shown by SDS-PAGE or Western blot using antibodies against the histidyl tag (Fig. 1). ZntA was localized in the membrane fraction of the cells.

Everted membrane vesicles prepared from cells of LMG194 (zntA::kan) expressing ZntA was accumulated 65Zn(II) in the presence of MgATP (Fig. 2A), indicating that ZntA was functionally expressed. 65Zn(II) accumulation was not observed in either uninduced cells or with induced cells of the disrupted strain without the pZntA plasmid. The specific rate of 65Zn(II) accumulation was much higher compared with the value observed previously for the wild-type strain, W3110, induced with 1 mM ZnSO4 (12); this result is consistent with ZntA being overexpressed upon induction of pZntA.

1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; BioTris, biss(2-hydroxyethyl)iminostrihydroxymethylmethane.
Solubilization and Purification of ZntA—A variety of detergents, including Triton X-100, Tween 80, n-octyl-β-D-glucopyranoside, dodecyl-β-D-maltoside, and sodium deoxycholate, were used for solubilization of ZntA from membrane suspensions. Triton X-100 was the most effective detergent; it was able to solubilize >90% of ZntA from the membranes as observed by Western blot (Fig. 1). Sodium deoxycholate, Tween 80, octylglucoside, and dodecylmaltoside were able to solubilize only a portion of the protein. ZntA solubilized with Triton X-100, octylglucoside, or dodecylmaltoside was purified in a single step using Ni(II) affinity chromatography. Of these three detergents, maximal ATPase activity was obtained with the Triton X-100-solubilized and purified protein. Therefore, in the optimized purification protocol, ZntA was solubilized from the membranes with 1% Triton X-100 and purified on a Ni(II) affinity column in the presence of 0.5% Triton X-100. Purified ZntA was stored in 0.2% Triton X-100. The presence of phospholipids during the purification had no effect on the activity of the purified enzyme.

As shown in Fig. 1, SDS-PAGE revealed that ZntA was >95% pure. The enzyme appeared to lose its activity upon storage by oxidation but could be reactivated by treatment with dithiothreitol or β-mercaptoethanol prior to assays.

Soft Metal Cation-dependent ATPase Activity of ZntA—Purified ZntA showed high levels of metal ion-dependent ATPase activity in the presence of phospholipids and Triton X-100. No ATPase activity was observed in the absence of phospholipids. Asolectin and E. coli phospholipids were both effective at stimulating activity. Treatment of the purified, detergent-solubilized protein with reducing agents was necessary in order to obtain the maximal activity. ZntA was active in the temperature range 25–45 °C, with the activity being 2-fold higher at 40 °C compared with 30 °C. Assays were routinely performed at 37 °C. High levels of activity were observed only in the presence of both Mg(II) as well as Pb(II), Cd(II), Hg(II), or Zn(II). The basal level of ATPase activity in the absence of soft metal cations was typically <10% of the metal cation-stimulated value. Fig. 3 shows the ATPase activity obtained as a function of the MgATP concentration in the presence and absence of 100 μM Pb(II) at pH 6.5 and 37 °C. The activity was highest for a 1:1 complex of Mg(II) and ATP; excess Mg(II) had no effect on the activity. The Pb(II)-dependent activity of ZntA had a K₉ values in Table I thus refer to those of soft metal ATP complexes.

Effect of Cysteine on the Soft Metal Ion-dependent ATPase Activity of ZntA—The lack of Cd(II)-stimulated activity of ZntA at pH 7.0 was surprising since ZntA mediates resistance of E. coli to toxic concentrations of Cd(II). Therefore, the effect of Cd(II) on ZntA was investigated further. It was observed that the addition of Cd(II) to the assay buffer completely inhibited the

**Fig. 1.** A, expression and purification of ZntA analyzed by 8% SDS-PAGE stained with Coomassie Blue. Lane 1, membrane fraction of E. coli strain LMG194 zntA::kan; lane 2, membrane fraction of LMG194 zntA::kan transformed with pZntA; lane 3, membrane fraction of LMG194 zntA::kan + pZntA, induced with 0.02% L-arabinose; lane 4, membrane fraction from lane 3 that was not solubilized with 1% Triton X-100; lane 5, Triton X-100 extract of the membrane fraction from lane 3; lane 6, purified, desalted ZntA; lane 7, molecular weight markers. The arrow denotes the band corresponding to ZntA. B, Western blot of the SDS-PAGE shown in A, with an antibody against the histidyl tag.
FIG. 3. The ATPase activity of ZntA as a function of the MgATP concentration. Assays were carried out at pH 6.5 and 37 °C, in the presence of 100 μM free Pb(II) (○) or in the absence of any soft metal (○). The assay buffer used was 0.1 M acetic acid, 0.05 M BisTris, and 0.05 M triethanolamine. The lines are fits to the Michaelis-Menten equation, \( V = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \). The MgATP concentration was calculated at each point from the total Mg(II) and ATP concentrations as detailed under “Experimental Procedures.”

FIG. 4. The ATPase activity of ZntA as a function of the soft metal cation concentrations in the absence and presence of thiolates. Assays were carried out at pH 7.0 and 37 °C, in the presence of 5 mM each Mg(II) and ATP. The same buffer was used as in Fig. 3. The lines are fits to the same equation as in Fig. 3. A, activity as a function of metal cation concentrations. ▲, Pb(II) (○); ○, Zn(II) (△); ●, Cd(II) (●). B, activity as a function of metal dithiolate concentrations. ▲, Pb(thiolate)\(_2\); ○, Zn(thiolate)\(_2\); ●, Cd(thiolate)\(_2\).

Pb(II)-induced ATPase activity of ZntA at pH 7.0. This inhibition could be protected by adding cysteine to the assay buffer. Moreover, ZntA displayed Cd(II)-stimulated ATPase activity at pH 7.0 when cysteine or glutathione was included in the assay medium. The effect of cysteine or glutathione was investigated in more detail at pH 7.0. For each of the three metals, Pb(II), Cd(II), and Zn(II), the activity increased with increasing concentrations of the unprotonated (thiolate) form of cysteine or glutathione until a maximum was reached for a metal ion: thiolate ratio of 1:2. The ATPase activities obtained as a function of the Pb(thiolate)\(_2\), Cd(thiolate)\(_2\), and Zn(thiolate)\(_2\) concentrations at pH 7.0 are shown in Fig. 4B. Pb(II), Cd(II), Zn(II), and other soft metals form complexes with thiolates with affinities that are typically many orders of magnitude greater than complexes with ATP (28). Therefore, unlike Fig. 4A where the soft metals are primarily present as lower affinity complexes with ATP, they are almost completely chelated with cysteine in Fig. 4B. A comparison of panels A and B in Fig. 4 shows that the \( V_{\text{max}} \) is greater when the metals are present as thiolate complexes. This effect is the most dramatic for Cd(II). The maximal activities and \( K_m \) values for Pb(thiolate)\(_2\), Cd(thiolate)\(_2\), and Zn(thiolate)\(_2\) are summarized in Table I. The \( K_m \) values were typically 10–20-fold higher for the dithiolate complexes of the metals.

Effect of Thiolates on the ATP-dependent Transport of \(^{109}\text{Cd(II)}\) at pH 7.0—Transport assays were performed with everted membrane vesicles prepared from pZntA-transformed LMG194 (zntA::kan) cells at pH 7.0 with 20 μM \(^{109}\text{Cd(II)}\) in the presence and absence of cysteine (Fig. 2B). When cysteine was absent, \(^{109}\text{Cd(II)}\) was accumulated at very low levels and showed saturation after 2–4 min. In the presence of cysteine (thiolate present at 2-fold the concentration of Cd(II)), \(^{109}\text{Cd(II)}\) accumulation was >5-fold higher and increased in a linear manner during the time scale of the measurement.

pH Dependence of the Soft Metal Ion-stimulated Activity, in the Absence and Presence of Cysteine—The pH dependence of the ATPase activity of ZntA was investigated in more detail. Fig. 5A shows the pH profiles for Pb(II), Cd(II), and Zn(II) in the pH range 5.5–8.0, in the absence of thiolates. For each pH, the activities were measured at two high metal ion concentrations (100 and 200 μM), to ensure that the maximal activity was obtained. The pH profiles for Pb(II) and Zn(II) are similar, with a pH optimum of 6.0–6.5. The activities decreased rapidly below pH 6.0 and above pH 7.5. The pH profile with Cd(II) was rather different, with essentially no ATPase activity observed above pH 6.5; this result is consistent with our earlier observation that Cd(II) inhibits ZntA at pH 7.0.

Fig. 5B shows the pH profile of the ATPase activity of ZntA in the presence of Pb(thiolate)\(_2\), Cd(thiolate)\(_2\), and Zn(thiolate)\(_2\). The activities were measured at a metal concentration of 600 μM. At each pH, sufficient cysteine was added to maintain the thiolate ion at a 2-fold excess over the metal ion concentration. For both Pb(II) and Zn(II), the ATPase activity of ZntA was elevated when thiolates were present in the assay buffer. In the case of Cd(II), ZntA showed high levels of activity at neutral pH in the presence of thiolates, with a pH profile that closely resembled that of Zn(II). This result is in striking

![Table I](image)

**Table I**

|                | \( V_{\text{max}} \) (μmol · mg \(^{-1} \) · min \(^{-1} \)) | \( K_m \) (μM) |
|----------------|-----------------------------------------------------------|----------------|
| Pb(II)         | 0.88 ± 0.01                                              | 6.9 ± 0.6      |
| Pb(thiolate)\(_2\) | 3.02 ± 0.22                                              | 115 ± 25       |
| Zn(II)         | 0.19 ± 0.01                                              | 5.1 ± 0.9      |
| Zn(thiolate)\(_2\) | 0.96 ± 0.02                                              | 109 ± 6        |
| Cd(II)         | Not detected                                              |                |
| Cd(thiolate)\(_2\) | 1.16 ± 0.05                                              | 115 ± 16       |
E. coli suggesting that ZntA does not contribute to Hg(II) resistance in disrupted strain showed similar levels of sensitivity to Hg(II), Cd(II). It is to be noted that wild-type E. coli characterized in detail. However, 100 μM Hg(thiolate)2; L, Zn(thiolate)2; ○, Pb(thiolate); ▲, Zn(thiolate)2; ●, Cd(thiolate)2.

contrast to the total lack of activity with Cd(II) above pH 6.5 when thiolates were absent. Due to the large concentrations of cysteine that were required at low pH levels, activities were not measured below pH 6.5.

The Metal Ion-stimulated ATPase Activity of ZntA Is Specific for Pb(II), Cd(II), Zn(II), and Hg(II)—Metal cations other than Cd(II), Pb(II), and Zn(II) were tested in the presence and absence of thiolates for their ability to stimulate the ATPase activity of purified ZntA. The “hard” metals, Ca(II), Na(I), and Ba(II), were ineffective at stimulating any ATPase activity. The “soft” metals, Co(II), Ni(II), Cr(III), Cu(I), Cu(II), SbO2 and AsO2, also did not show any ATPase activity. Surprisingly, ZntA displayed Hg(II)-induced ATPase activity at pH 7.0 in the presence of added thiolates. In the absence of added thiolates, Hg(II) is a potent inhibitor of the coupled assay system used in this study; hence, the Hg(II) activity of ZntA was not characterized in detail. However, 100 μM Hg(thiolate)2 stimulated the ATPase activity of ZntA to the same extent as 100 μM Cd(thiolate)2 at pH 7.0, indicating that Hg(II) is at least as effective as Cd(II). It is to be noted that wild-type E. coli and the zntA-disrupted strain showed similar levels of sensitivity to Hg(II), suggesting that ZntA does not contribute to Hg(II) resistance in E. coli (12).

**DISCUSSION**

Soft metal transporting P-type ATPases, presently numbering more than 30 pumps, are a rapidly growing subgroup of the large family of P-type ATPases. With the discovery of the human Menkes and Wilson disease-associated proteins, which are responsible for copper uptake and homeostasis (5, 6), much attention has been focused on these ATPases. ATP-dependent transport of metal cations has been demonstrated for both the Cu(I) and Zn(II)/Cd(II) pumps (8, 12–15, 18). The soft metal ATPases have a distinctive metal-binding motif in the amino-terminal domain. Recently, the ability of the isolated NH2-terminal domains of the Menkes and Wilson disease-related proteins to bind metal ions have been shown (16, 17) and the structure of the isolated domain solved (20). The formation of a phosphorylated intermediate has been shown for CopB from E. hirae, CadA from S. aureus, and recently for the Menkes protein (21, 30, 31). However, there have been no convincing reports of soft metal cation-dependent ATPase activity for the P1-type ATPases. CopB from E. hirae is the only other soft metal ATPase that has been purified. Very low levels of ATPase activity were measured for CopB; however, it showed no stimulation of activity upon addition of Cu(I) (21). It was recently reported that microsomes from mouse liver contain a P-type ATPase. The microsomes also exhibit copper and iron-stimulated ATPase activity (22). However, the optimal ATPase activity obtained was rather low, ~16 nmol/(mg·min); this activity could not be assigned to a specific protein.

ZntA, the product of o732, is one of two soft metal translocating ATPases in E. coli. ZntA was previously shown to confer bacterial resistance toward toxic concentrations of Zn(II), Cd(II), and Pb(II) (12, 13). Everted membrane vesicles were shown to catalyze ATP-dependent transport of 65Zn(II) and 109Cd(II). Our objectives in this study were to express zntA under a heterologous promoter, purify the protein, and characterize its soft metal-dependent ATP hydrolysis activity. An ATPase assay on purified ZntA would be a convenient and quantitative way to study the mechanism of the P1-type ATPases.

In this work, we report the expression of histidyl-tagged ZntA in a functional form under control of the tightly regulated araBAD promoter in a zntA-disrupted strain. The expressed ZntA was solubilized from membranes with Triton X-100 and purified to homogeneity on an affinity column. Purified, detergent-soluble ZntA showed ATPase activity that was dependent on the presence of Mg(II) as well as divalent soft metal cations. The maximal activity obtained was ~3 μmol/(mg·min) in the presence of a Pb(II)-thiolate complex; in the absence of soft metal, the activity was ~10-fold lower. This is the first demonstration of high levels of soft metal-stimulated ATPase activity for a purified P1-type ATPase.

ZntA hydrolyzed ATP only in the presence of Mg(II); the K_M for MgATP was ~110 μM. Pb(II) and Zn(II) stimulated the ATPase activity at neutral pHs, with the Pb(II)-dependent activity being ~4-fold higher than that of Zn(II) at optimal pH. The K_M values for Pb(II) and Zn(II) were very similar, 5–10 μM; these K_M values reflect those of the ATP complexes of Pb(II) and Zn(II). Thus, ZntA is more efficient as a Pb(II)-ATPase than a Zn(II)-ATPase. Surprisingly, Cd(II) appeared to inhibit ZntA above pH 6.0; however, Cd(II)-thiolate complexes protected this inhibition and also stimulated the ATPase activity. For Pb(II) and Zn(II), the activity was 3–4-fold higher for thiolate complexes of these metals; for Cd(II), this effect was much more dramatic in that no activity was observed for the non-thiolate complex of Cd(II) above pH 6.5. A 1:2 complex of metal cation to thiolate was the most efficient substrate at pH 7.0. The only other metal that stimulated the ATPase activity of ZntA was Hg(II); the diethyl complex of Hg(II) was as effective as those of Zn(II) and Cd(II) at neutral pH. No activity was observed with Cu(I) or Cu(II) in the presence or absence of thiolates. Our data thus indicate that ZntA is a *divalent soft* metal cation ATPase, as opposed to the bacterial and eukaryo-
The ATPase Activity of a Pb(II)/Cd(II)/Zn(II) Pump

We have previously shown that CadC, the transcriptional regulator of CadA, responds to soft metal cations and mediates resistance to Pb(II), Cd(II), and Zn(II), with the highest activity obtained in the presence of Pb(II). With a purification protocol established, as well as transport and ATPase assays optimized, ZntA is an ideal system to study the mechanisms of soft metal recognition and transport in the P1-ATPases.

Note Added in Proof—While this manuscript was in press, an article by J. Okkeri and T. Haltia was published describing the overexpression of ZntA in E. coli and its soft metal-stimulated ATPase activity in membrane fractions (1999). Our results are in qualitative agreement with those presented in this article; however, the metal specificity of purified ZntA that we have described here is slightly different from that of ZntA in membrane fractions as shown by these authors. This discrepancy may be due to the presence of the second soft metal P-type ATPase of E. coli in their membrane preparations. We have recently shown that this second ATPase is a Cu(II)/Ag(I)-transporting ATPase (Rensing, C., Fan, B., Sharma, R., Mitra, B. & Rosen, B. P. (2000) Proc. Natl. Acad. Sci. U.S.A., in press).

Although ZntA mediates resistance of E. coli to Zn(II), Cd(II), and Pb(II), Cd(II) inhibited the enzyme at physiological pHs; for Zn(II) and Pb(II), the ATPase activities were 3–4-fold higher in the presence of thiolate complexes compared with the lower affinity complexes with ATP. Physiologically, the concentrations of the free soft metal ions are expected to be very low; it has been recently suggested that free intracellular Cu(I) in yeast is negligible (32). The divalent soft metals, Pb(II), Zn(II), Cd(II), and Hg(II), are likely to be present inside cells as complexes of glutathione (32). The divalent soft metals, Pb(II), Zn(II), Cd(II), and Hg(II), are likely to be present inside cells as complexes of glutathione or may be tightly bound to as yet unidentified "chaperone" proteins similar to those for Cu(I) (32, 33). Therefore, it is very likely that the physiological substrates for the ATPase activity of ZntA are the metal thiolate complexes.

ZntA as well as CadA from S. aureus, a close homolog of ZntA, mediate resistance to Pb(II), Cd(II), and Zn(II) (12–14). We have previously shown that CadC, the transcriptional regulator of CadA, responds to soft metal cations in the order Pb(II) > Cd(II) > Zn(II) (13). In this study, we show that soft metal cations stimulate the ATPase activity of ZntA in the order Pb(II) > Cd(II) > Zn(II) > Hg(II). These observations suggest that the primary physiological function of ZntA, CadA, and their close homologs is to mediate bacterial resistance to the highly toxic metals, Pb(II) and Cd(II). A putative eukaryotic ZntA homolog has been identified in A. thaliana, suggesting that plants may employ similar resistance mechanisms. Mammalian ZntA homologs may exist, although none has been identified yet.

In conclusion, ZntA from E. coli has been expressed, solubilized, and purified. ZntA shows high levels of soft metal cation-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). With a purification protocol established, as well as transport and ATPase assays optimized, ZntA is an ideal system to study the mechanisms of soft metal recognition and transport in the P1-ATPases.

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