The Metal Specificity and Selectivity of ZntA from *Escherichia coli* Using the Acylphosphate Intermediate*

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*This work was supported by United States Public Health Service Grant GM-61689 (to B. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ZntA from *Escherichia coli* is a P-type ATPase that confers resistance to Pb(II), Zn(II), and Cd(II) in *vivo*. We had previously shown that purified ZntA shows ATP hydrolysis activity with the metal ions Pb(II), Zn(II), and Cd(II). In this study, we utilized the acylphosphate formation activity of ZntA to further investigate the substrate specificity of ZntA. The site of phosphorylation was Asp-436, as expected from sequence alignments. We show that in addition to Pb(II), Zn(II), and Cd(II), ZntA is active with Ni(II), Co(II), and Cu(II), but not with Cu(I) and Ag(I). Thus, ZntA is specific for a broad range of divalent soft metal ions. The activities with Ni(II), Co(II), and Cu(II) are extremely low; the activities with these non-physiological substrates are 10–20-fold lower compared with the values obtained with Pb(II), Zn(II), and Cd(II). Similar results were obtained with a ZntA derivative lacking the amino-terminal metal binding domain. By characterizing the acylphosphate formation reaction in ZntA in detail, we show that a step prior to enzyme phosphorylation, most likely the metal ion binding step, is the slow step in the reaction mechanism in ZntA. The low activities with Ni(II), Co(II), and Cu(II) are because of a further decrease in the rate of binding of these metal ions. Thus, metal ion selectivity in ZntA and possibly other P1-type ATPases is based on the charge and the ligand preference of particular metal ions but not on their size.

P-type ATPases are transporters that utilize the energy liberated in the exergonic ATP hydrolysis reaction to translocate positively charged substrates across membranes (1). Their name derives from the fact that they form an acylphosphate intermediate during the reaction cycle, in which the γ-phosphate of ATP is transferred to a conserved aspartate residue. Thus, P-type ATPases are evolutionarily distinct from other families of ATP-dependent pumps; mechanistically, they belong to the halocid dehalogenase protein superfamily (2). P-type ATPases have been divided into five subgroups; one such subgroup, the P1-type ATPases found in archaea, bacteria, and eukarya, transport soft metal cations such as Cu(I), Ag(I), Zn(II), Cd(II), Pb(II), and Co(II) (3–6). The physiological functions of these pumps include maintaining the homeostasis of essential metals such as Cu(I), Co(II), and Zn(II), as well as mediating resistance to toxic concentrations of Pb(II), Cd(II), Cu(I), and Ag(I). These pumps may also be involved in delivering essential trace metal ions to target enzymes.

P1-type ATPases first became the focus of attention when it was discovered that two human proteins, implicated in Menke's and Wilson's diseases, were Cu(I) transporters (7–9). Cu(I) transporters have also been characterized in bacteria and yeast; in addition to Cu(I), some of these pumps have been shown to recognize Ag(I) as a substrate cation (10–17). Besides the Cu(I)/Ag(I) transporters, other P1-type ATPases have been characterized that transport soft metal ions such as Pb(II), Cd(II), Zn(II), and Co(II); to date, these divalent soft metal transporters have been found in bacteria and plants (18–20). The best characterized transporter from this latter group is ZntA from *Escherichia coli*, a protein that mediates resistance to toxic concentrations of Pb(II), Cd(II), and Zn(II). Both the Cu(I)/Ag(I) and the Pb(II)/Zn(II)/Cd(II) P1-type ATPases have a distinctive, highly polar metal-binding amino-terminal domain containing 1–6 repeats of the conserved sequence GXXCXXC, as well as the conserved CPC motif in the sixth transmembrane helix that is believed to be part of the translocation pathway. In fact, all P1-type pumps are highly homologous to each other; hence, the basis of metal ion specificity in these pumps remains an intriguing question.

Before we attempt to understand how substrate specificity is determined in P1-type ATPases, we need to establish the full spectrum of all the metal ions that are recognized as substrates by these pumps. We have previously shown that the physiological substrates for ZntA are Pb(II), Zn(II), and Cd(II) (18, 21). Also, purified ZntA shows ATP hydrolysis activity only in the presence of these three metal ions (22). However, it was reported that the monovalent ion, Ag(I), induces ATP hydrolysis but not the partial reaction of acylphosphate formation in ZntA (22). In this study our goal was to establish the full substrate spectrum of purified ZntA as well as gain further insights into the basis of metal ion selectivity in ZntA. We reexamined the substrate specificity of ZntA using the acylphosphate formation assay; because this assay has a much lower background activity, it is more sensitive compared with the ATP hydrolysis assay. Our results clearly showed that ZntA forms the acylphosphate intermediate not only with the physiological substrates, Pb(II), Zn(II), and Cd(II), but also the divalent metal ions, Co(II), Cu(II), and Ni(II). However, no activity was seen with the monovalent ions Ag(I) and Cu(I). Studies with the D436N mutant, in which the site of phosphorylation, Asp-436, was altered, showed that this broader metal usage was indeed because of ZntA and not because of an artifact. Additionally, ATP hydrolysis activity could be detected with these same divalent metals in pulse-chase experiments with non-radioactive ATP, though the activity with these metals is clearly rather low compared with the metal ions that are the physiological substrates. No ATPase activity was detected with
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Detailed characterization of the acylphosphate intermediate formation was carried out with two different mutant proteins expressing recombinant ZntA were able to form the acylphosphate intermediate in the presence of ATP and the metal ions (22). The sensitivity of the acylphosphate intermediate to sodium vanadate substrates of ZntA (Fig. 1A). The monovalent metal ions, Ag(I) and Cu(I), were unable to stimulate intermediate formation in ZntA were purified as described before (24). Phosphorylation of the purified proteins with \([\gamma-32P]ATP\) was carried out in 100 \(\mu\)l of reaction mixture containing 50 \(\mu\)M Tris-Cl, pH 7.0, 0.1% asolectin, 10% glycerol, 4 \(\mu\)g of pure protein pretreated with 2 \(\mu\)l diethiothreitol, and 50 \(\mu\)l of appropriate metal-salt solution. The reaction mixture was incubated at room temperature for 10 min and the reaction initiated with 10 \(\mu\)M MgCl\(_2\) and 2.5 \(\mu\)Ci of \([\gamma-32P]ATP\) (final concentration, 1 \(\mu\)M) at room temperature or 37 °C. After 20 s, the reaction was stopped with 10 \(\mu\)l of 10% ice-cold trichloroacetic acid and the samples were incubated on ice for 10 min and then centrifuged for 10 min. The pellet was washed four times with 10% ice-cold trichloroacetic acid and 1 \(\mu\)M NaH\(_2\)PO\(_4\) followed by resuspension in acidic buffer (25 \(\mu\)M H\(_2\)PO\(_4\), pH 2.4, 5% SDS). Blank samples were obtained by adding stop solution before the addition of \([\gamma-32P]ATP\). Aliquots were used for scintillation counting.

To measure the kinetics of dephosphorylation, purified proteins were first phosphorylated for 20 s at room temperature in the presence of 30 \(\mu\)M metal-salt solution and then incubated with either 10 \(\mu\)M EDTA or 10 \(\mu\)M EDTA together with 2 \(\mu\)M ADP on ice. At fixed time intervals, the reactions were stopped with 10 \(\mu\)M NaH\(_2\)PO\(_4\), centrifuged, and worked up as described earlier.

Acylphosphate Formation with Purified Proteins—Everted Membrane Vesicles with \([\gamma-32P]ATP\) Reactions were carried out with membranes containing \(\approx 50\ \mu\)g of total protein in 100 \(\mu\)l of 20 mM BisTris propane, pH 6.0, containing 200 mM KCl, 10 \(\mu\)M EDTA, and 0.1% asolectin at room temperature for 5 min, followed by the addition of either 3 \(\mu\)l of \(\text{H}_2\text{O}\) or 1 \(\mu\)l metal salt solution. Following a further incubation for 5 min, the reaction mixture was initiated with 10 \(\mu\)l of 100 \(\mu\)M MgCl\(_2\) (final concentration, 9 \(\mu\)M) and 2 \(\mu\)l of \([\gamma-32P]ATP\) (final concentration, 20 \(\mu\)M). After 2 min, the reaction was stopped with 500 \(\mu\)l of 10% cold trichloroacetic acid containing 1 \(\mu\)l NaH\(_2\)PO\(_4\), incubated on ice for 10 min and centrifuged for 10 min, in a microcentrifuge. The pellet was washed four times with 10% ice-cold trichloroacetic acid containing 1 \(\mu\)l NaH\(_2\)PO\(_4\), and 1 \(\mu\)l of 10% cold trichloroacetic and 1 \(\mu\)l of 10% ice-cold trichloroacetic acid followed by 1 \(\mu\)l of 10% cold trichloroacetic acid. The sensitivity of the acylphosphate intermediate to sodium vanadate was measured by incubating 4 \(\mu\)g of purified protein with different concentrations of vanadate together with 2 \(\mu\)M MgCl\(_2\) and 50 \(\mu\)M lead acetate for 10 min at 37 °C prior to initiating the reaction with 1 \(\mu\)l \([\gamma-32P]ATP\) and MgCl\(_2\) (final concentration, 8 mM). The reaction mixture was quenched after 10 s with 10% cold trichloroacetic acid, and was centrifuged, and the pellet resuspended as described earlier. Aliquots were used for scintillation counting.

Protein concentrations were determined using the bicinchoninic acid reagent with bovine serum albumin as standard.

**RESULTS**

Metal Specificity of Acylphosphate Formation in Membrane Vesicles—As expected for a P-type ATPase, membrane vesicles expressing recombinant ZntA were able to form the acylphosphate intermediate in the presence of ATP and the metal ions Pb(II), Cd(II), and Zn(II) that were previously shown to be the substrates of this transporter (Fig. 1A). Surprisingly, when other metal salts were tested, we observed that ZntA was able to form the intermediate in the presence of Co(II), Cu(II), and Ni(II), divalent metal ions not previously identified as substrates of ZntA (Fig. 1A). The monovalent metal ions, Ag(I) and Cu(I), were unable to stimulate intermediate formation in ZntA. No acylphosphate intermediate was observed in the absence of metal ions. The substrate specificity of intermediate formation for \(\Delta N\)-ZntA, a ZntA mutant lacking the metal-binding amino-terminal domain, was identical to that of ZntA (Fig. 1B). It is to be noted that different concentrations of

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1 The abbreviations used are: \(\Delta N\)-ZntA, a mutant of ZntA with residues 2-106 deleted; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-hydroxyethylpropane-1,3-diol.
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Phosphorylated protein were loaded in Fig. 1A such that the total radioactivity in each lane is the same; in Fig. 1B, the same concentration of protein is loaded in each lane and illustrates the different levels of acylphosphate formed for the different metals.

To eliminate the possibility that the broader substrate specificity was an artifact caused by using crude membrane vesicles, we tested the ability of different metals to stimulate the intermediate formation in membrane vesicles expressing recombinant ZntA. The same amount of quenched reaction mixture was loaded in each lane. For the last three lanes, an excess reaction mixture was loaded to detect any phosphorylated proteins. B, membrane vesicles prepared from LMG194 (zntA:kan) expressing recombinant ZntA. The same amount of quenched reaction mixture was loaded in each lane. C, membrane vesicles prepared from LMG194 (zntA:kan) expressing recombinant ΔN-ZntA or the D436N mutant.

Metal Specificity and Selectivity of ZntA in the Membranes—We previously showed that purified ZntA shows detectable ATP hydrolysis activity only with Pb(II), Zn(II), and Cd(II); no activity was observed with Co(II), Cu(II), and Ni(II) (24). We examined the metal specificity of the ATPase activity of crude ZntA in membrane vesicles by measuring the phosphate release activity using a discontinuous colorimetric assay (data not shown). Activity was obtained with Pb(II), Zn(II), and Cd(II); however, activity could not be detected with Co(II), Cu(II), and Ni(II) above that of background levels. This raised the possibility that the metal ions Co(II), Cu(II), and Ni(II) were only able to stimulate the partial reaction of acylphosphate formation in ZntA and not total turnover. Because the level of background activity is low for acylphosphate formation in membranes given that there are only four P-type ATPases in E. coli, we next used this method to assay for overall turnover in the presence of these metal ions. Pulse-chase experiments were performed where following formation of the acylphosphate intermediate in the presence of different metal ions, its decay was monitored after the addition of non-radioactive ATP (Fig. 2). The results clearly show that the intermediate disappeared rapidly upon addition of non-radioactive ATP for all the metal ions tested. Because the disappearance of the intermediate in the presence of ATP results from enzyme turnover, it is clear that Ni(II), Cu(II), and Co(II) are also substrates of ZntA, though with very low activities that typically cannot be detected above background levels when the hydrolysis activity is directly assayed.

Steady-state Levels of Acylphosphate Intermediate for ZntA with Different Metals—We next investigated the basis of metal selectivity in ZntA, that is the reason why the overall activity with Cu(II), Ni(II), and Co(II) appeared to be much less than with Pb(II), Zn(II), and Cd(II). Fig. 3 shows the steady-state levels of the acylphosphate intermediate formed by ZntA in membrane vesicles for all six metal ions. Steady-state levels were reached within 10–20 s for all six metal ions (data not shown). Maximal steady-state levels of the phosphorylated enzyme were accumulated for Cd(II) followed by Zn(II), Pb(II), and Cu(II). Phosphorylated enzyme levels for Co(II) and Ni(II) are much lower. The data suggest that the extremely low ATPase activity of ZntA with Cu(II), Ni(II), and Co(II) is not because of decreased rates of the steps in the reaction following acylphosphate formation but rather because of decreased rates of one or more steps prior to acylphosphate formation. In fact, steps following acylphosphate formation are relatively slow for Cd(II), and to a lesser extent for Zn(II), metal ions that are fairly good substrates (Scheme 1). This conclusion is also supported by Fig. 2, which shows that the acylphosphate intermediate formed with Cd(II) and Zn(II) disappears at the slowest rates when excess cold ATP is added.

Acylphosphate Formation with Purified Proteins—We next characterized the intermediate formed with purified protein in
greater detail. Both purified ZntA and ΔN-ZntA, pretreated with dithiothreitol, were able to form the acylphosphate intermediate in the presence of ATP and the six different metal salts. Thus, both purified, detergent-soluble protein as well as native protein in membrane vesicles exhibit activity with a broad range of divalent metal ions. Steady-state levels of the intermediate in the presence of different metal salts were determined at room temperature by measuring time courses of intermediate formation (data not shown). The steady-state level of the intermediate was 10-20-fold lower for Ni(II), Co(II), and Cu(II) compared with Pb(II), Zn(II), and Cd(II) for both ZntA and ΔN-ZntA. Interestingly, in contrast to the case with ZntA in the membranes, the lowest level of intermediate formed for purified ZntA was in the presence of Cu(II); this is most likely because of the reduction of most of the Cu(II) to Cu(I) by dithiothreitol present in the assay mixture.

The dependence of intermediate formation on metal ion concentration was measured for Pb(II), Zn(II), and Cd(II), the three most active substrate metals (data not shown). The $K_m$ values for Pb(II), Zn(II), and Cd(II) were 6.4 ± 1.6, 5.4 ± 1.4, and 4.2 ± 1.4 μM, respectively. These values are similar to the $K_m$ values previously obtained for the steady-state ATPase activity of purified ZntA, 5.9 ± 0.4, 5.2 ± 0.7, and 3.8 ± 0.8 μM for Pb(II), Zn(II), and Cd(II), respectively (27). $K_m$ values for Ni(II), Co(II), and Cu(II) could not be determined accurately because of the extremely low levels of intermediate formation at low metal concentrations. The dependence of intermediate formation for purified ZntA on the concentration of ATP was measured in the presence of Pb(II); the $K_m$ for ATP was 2.6 ± 0.8 μM (data not shown). In contrast, the $K_m$ obtained previously for the steady-state ATP hydrolysis activity of purified ZntA was 106 ± 13 μM at pH 7.0 and 37 °C (27).

As expected for the acylphosphate intermediate formed during the reaction of a P-type ATPase, it was sensitive to vanadate, a classic inhibitor of P-type ATPases. We measured the inhibition of the intermediate formation by vanadate in both crude membrane preparations as well as with purified protein. In crude membrane preparations, we obtained an IC50 of 1.5 mM (data not shown). This value is considerably higher than corresponding values for P2-type ATPases. Similar observations have been reported for other P1-type ATPases (28–30). However, the IC50 obtained for the inhibition of the intermediate formation by vanadate for purified ZntA was 50 μM (Fig. 4A). This value is similar to the IC50 of <20 μM, obtained for the vanadate inhibition of the ATP hydrolysis activity of purified ZntA (Fig. 4B). Thus, vanadate appears to be as potent an inhibitor of ZntA as has been observed for P2-type ATPases; the weaker inhibition observed in membranes is possibly an artifact of using crude membrane preparations that have other P-type ATPases in addition to ZntA.

**Kinetics of Dephosphorylation of the Acylphosphate Intermediate with EDTA and ADP**—The decay of the acylphosphate intermediate upon addition of ATP indicated that during overall turnover, the steps following intermediate formation were slowest for Cd(II) and Zn(II) but not for Pb(II) or the slow metal substrates, Ni(II), Co(II), and Cu(II). To confirm this result...
directly, we measured the rates of dephosphorylation of the acylphosphate intermediate formed by ZntA in the presence of EDTA or EDTA and ADP for the three most active metal substrates. As shown in Fig. 5 A, the rate of dephosphorylation in the presence of a strong metal chelator alone, representing dephosphorylation from the E2P state (Scheme 1), is much faster for the intermediates formed with Zn(II) and Pb(II) than with Cd(II). However, the rate of dephosphorylation in the presence of ADP and EDTA, representing dephosphorylation from both the E1P and E2P states, is extremely rapid for the intermediates formed with all three metal ions (Fig. 5 B). These data suggest that a similar fraction of the intermediate is present in each conformational state for Pb(II) and Zn(II). However, a slightly larger fraction of the intermediate is in the E1 state for Cd(II) relative to Pb(II) and Zn(II), and the E3P to E2P conformational change in ZntA may be partially rate-limiting with Cd(II) as the substrate.

**Acylphosphate Formation with [32P]P**—P-type ATPases can form the acylphosphate intermediate with inorganic phosphate via phosphorylation of the E2 state (Scheme 1). Both purified ZntA and ∆N-ZntA could be phosphorylated using inorganic phosphate in the absence of metals. The presence of metal ions, which bind to the E1 conformational state, is expected to favor the equilibrium toward the E1 state and hence result in a lower level of phosphorylation by inorganic phosphate. However, we observed the presence of metal ions did not have a significant effect on the level of phosphorylation by inorganic phosphate for either ZntA or for ∆N-ZntA (data not shown). This result is in contrast to the conclusion drawn earlier with ZntA in membranes (23); however, in that study, results were not quantitated. The maximal amount of acylphosphate formed with ZntA with inorganic phosphate was 1.5 nmol/mg of protein at room temperature irrespective of whether metal ions were present or not. These results demonstrate that the rate of metal ion binding to the E1 state is slow relative to the rate of phosphorylation of the E2 state such that the presence of metals does not result in a significant fraction of the transporter being present in the E1 state.

**DISCUSSION**

Despite highly homologous sequences, P1-type ATPases that have been characterized to date have been shown to be specific for either Cu(I) and Ag(I), or for Pb(II), Zn(II), and Cd(II). ZntA is the best characterized divalent metal ion-dependent P1-type ATPase to date. We have previously shown that ZntA displays in vivo resistance and in vitro ATPase activity with Zn(II), Cd(II), and Pb(II) (18, 21). Unlike Cu(I) and Ag(I), substrates of the monovalent metal ion P1-type ATPases, Pb(II), Zn(II), and Cd(II) do not belong to the same group in the periodic table and have substantially different properties. Our long term goal is to understand the molecular basis of metal ion specificity and selectivity displayed by these pumps. One of our goals in this study was to determine whether the metal specificity of ZntA...
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and its homologues is confined strictly to the three metal ions observed previously or whether other divalent metal ions are also recognized by ZntA, albeit with lower selectivity. We also wanted to reexamine whether Ag(I) is a substrate for ZntA, as had been reported earlier (23). Given the low catalytic activities of P1-type ATPases that have been characterized until now, it is technically challenging to identify additional metal ion substrates that may have extremely low activities, especially using ATP hydrolytic assays. This is, in part, because of the high levels of background ATPase activities resulting from the presence of other types of ATPases typically present in membranes. P-type ATPases differ from F- and V-type ATPases in forming a covalent intermediate during the reaction cycle in which the γ-phosphate of ATP is transferred to a conserved aspartate residue (1). Because the E. coli F, Fγ, ATPase does not form the acylphosphate intermediate, this is a more sensitive assay to test for additional metal ion substrates with low activities when the protein is expressed in E. coli. In this study, using the acylphosphate formation assay, we showed that recombinant ZntA in membrane vesicles was able to utilize Co(II), Ni(II), and Cu(II) as substrates in addition to Pb(II), Cd(II), and Zn(II). Thus, the metal ion specificity is more broadly confined to divalent soft-metal ions than previously suspected. Our results also unequivocally ruled out monovalent metal ions such as Ag(I) and Cu(I) as substrates for ZntA. Similar results were also obtained with ΔN-ZntA, supporting our earlier conclusion that the cysteine-rich amino-terminal domain of ZntA is not required for function, although our data suggest that ZntA forms a stronger dimer when this domain is present (24). Results with the D436N mutant also showed that Asp-436 is the site of phosphorylation; the D436N mutant is completely inactive with respect to both in vivo resistance and ATP hydrolysis activity.

Though ZntA or ΔN-ZntA was capable of complete turnover in the presence of Co(II), Ni(II), or Cu(II), the ATPase activities with these metal ions are extremely low because we could not detect any activity above background levels when ATP hydrolysis was directly assayed. Consistent with this observation, we observed that a zntA-deleted strain did not display increased sensitivity to Ni(II), Co(II), or Cu(II); additionally, overexpressed ZntA did not confer resistance to toxic concentrations of Ni(II), Co(II), and Cu(II). Thus, in vivo, ZntA is selective for Pb(II), Zn(II), and Cd(II) only. Of the three physiological metal ion substrates, purified ZntA has the highest ATPase activity with Pb(II), followed by Zn(II) and Cd(II) (22). A goal of this study was to understand the basis of metal ion selectivity in ZntA in terms of the reaction mechanism (Scheme 1). Steady-state levels of acylphosphate formed with ZntA are highest for Cd(II) and quite low for the slow substrates, Co(II), Ni(II), and Cu(II). Because an increased level of accumulation of a reaction intermediate implies that steps beyond the formation of that intermediate are slow, our results clearly show that the low activities with Co(II), Ni(II), and Cu(II) are not because of slow steps following acylphosphate formation but possibly because of slow steps preceding acylphosphate formation. In general, the steady-state levels of acylphosphate formation for all six metal ions were quite low, suggesting that for ZntA, one or more steps preceding acylphosphate formation is rate-limiting for all the metal ion substrates; it is reasonable to assume that this is the metal binding step (k1 in Scheme 1). However, because comparatively the highest level of intermediate was accumulated for Cd(II), it is possible that a second step following acylphosphate formation may also contribute to the overall rate for this particular metal ion.

These conclusions are also supported by our data measuring dephosphorylation of the acylphosphate intermediate formed with different metal ions in the presence of either EDTA alone or EDTA and ADP. In the presence of EDTA alone, the intermediate decays from the E2 state. When ADP is present, E1P, but not E2P, can transfer the phosphate group to ADP; thus the intermediate decays from the E1P state (Scheme 1). The decay of the intermediate was quite fast via either pathway, supporting our proposal that none of the steps involved in the decay of the intermediate is slow. However, the intermediate formed with Cd(II) decays at a slightly slower rate via the E1P pathway, once again suggesting that for the reaction with Cd(II), a second step, possibly the E1P-E2P transition is partly rate-limiting. When the acylphosphate was formed with inorganic phosphate for purified ZntA and ΔN-ZntA, the presence of metal ions, which bind to the E1 state and push the equilibrium to the E2 state, did not affect the level of the intermediate. These results also support our conclusion that the rate of metal ion binding to the E1 state is slow relative to the rate of phosphorylation of the E2 state.

Interestingly, the level of acylphosphate intermediate accumulated for ΔN-ZntA was ~2-fold higher relative to ZntA. As noted in a previous study, ΔN-ZntA has a slightly lower ATPase activity relative to ZntA (24). It is possible that a catalytic step after phosphorylation but prior to dephosphorylation is slower in ΔN-ZntA compared with ZntA, for example, the E1P-E2P transition or metal ion release from E2P (Scheme 1). It is not clear, however, how removal of the amino-terminal metal-binding domain can lead to a slowing of either of these steps.

The Km values obtained with ZntA for Pb(II), Zn(II), and Cd(II) for the acylphosphate formation activity are similar to those we measured previously for the ATPase activity; all three metals have similar affinities that are in the low micromolar range. These similar Km values for both the first half-reaction and overall turnover further support our hypothesis that the slow step in the reaction occurs before acylphosphate formation. The Km for ATP obtained with ZntA for the acylphosphate intermediate activity is ~2.6 μM. Similar values have been obtained for CopA from Archaeoglobus fulgidus and the Wilson’s disease Cu(I)-ATPase (16, 31). In contrast, the Km for ATP obtained with ZntA for the ATP hydrolysis activity is ~106 ± 13 μM at pH 7.0 and 37 °C (27); a value of 250 μM was obtained for CopA from A. fulgidus (16). This difference in Km values is probably because of the two different affinities for ATP that have been demonstrated for P-type ATPases (1). The maximal amount of acylphosphate formed with ZntA under saturating ATP concentrations is 0.23 ± 0.02 nmol/mg of protein at room temperature in the presence of Pb(II). This value is ~5-fold lower than that obtained with CopA from A. fulgidus but similar to the plant plastid plasma membrane H+ -ATPase (16, 32). These low steady-state levels of acylphosphate for the P1-type ATPases again suggest that the rate-limiting steps occur prior to acylphosphate formation for these transporters.

CONCLUSION

In this study we show that ZntA from E. coli shows activity specifically with divalent soft metal ions; the monovalent ions, Cu(I) and Ag(I), are not substrates. Specificity toward divalent metal ions is broader than we previously suspected. Using the sensitive acylphosphate formation activity, we show that in addition to Pb(II), Zn(II), and Cd(II), three other metal ions, Ni(II), Co(II), and Cu(II), are also substrates of this pump, though with considerably lower activity. ΔN-ZntA, a ZntA mutant lacking the amino-terminal metal-binding domain, displays the same metal specificity and selectivity as ZntA. We provide evidence that the low activity with the latter three ions is most likely the result of decreased rates of metal ion binding. Our results also lend further support to our earlier hypothesis...
that metal ion binding steps are primarily rate-limiting for ZntA and perhaps other P1-type ATPases. Soft-metal ions are believed to bind to ZntA primarily through cysteine ligands (33). Thus, ZntA appears to discriminate between metal ions based on charge and also on the affinity of the particular metal ion for sulfur-containing ligands. However, in contrast to P2-type ATPases, the metal ion size appears not to be important in determining selectivity because Pb(II) and Cd(II) are much larger than Zn(II), Ni(II), Co(II), and Cu(II); the latter four have similar sizes and charge but display markedly different activities.

REFERENCES

1. Inesi, G. (1985) Annu. Rev. Physiol. 47, 573–601
2. Aravind, L., Galperin, M. Y., and Koonin, E. V. (1998) Trends Biochem. Sci. 4, 127–129
3. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
4. Soliz, M., and Vulpe, C. (1996) Trends Biochem. Sci. 21, 237–241
5. Axelsen, K. B., and Palmgren, M. G. (1996) J. Mol. Biol. 46, 84–101
6. Gatti, D., Mitra, B., and Rosen, B. P. (2000) J. Biol. Chem. 275, 34009–34012
7. Mercer, J. F., Livingston, J., Hall, B., Paynter, J. A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D., and Glover, T. W. (1995) Nat. Genet. 3, 20–25
8. Vulpe, C., Levinson, B., Whitney, S., Packman, S., and Gitschier, J. (1993) Nat. Genet. 3, 7–13
9. Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R., and Cox, D. W. (1993) Nat. Genet. 3, 327–337
10. Odermatt, A., Suter, H., Krapf, R., and Soliz, M. (1993) J. Biol. Chem. 268, 12775–12779
11. Fu, D., Beeler, T. J., and Dunn, T. M. (1995) Yeast 11, 283–292
12. Kanamaru, K., Kashiwagi, S., and Mizuno, T. (1994) Mol. Microbiol. 13, 369–377
13. Phung, L. T., Ajlani, G., and Haselkorn, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9651–9654
14. Melchers, K., Herrmann, L., Mauch, F., Bayle, D., Heuermann, D., Weizenecker, T., Schuhmacher, A., Sachs, G., Haas, R., Bode, G., Besche, K., and Schafer, K. P. (1998) Acta Physiol. Scand. Suppl. 643, 123–135
15. Bensing, C., Fan, B., Sharma, R., Mitra, B., and Rosen, B. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 652–656
16. Mandal, A. K., Cheung, W. D., and Arguello, J. M. (2002) J. Biol. Chem. 277, 7201–7208
17. Gupta, A., Matsui, K., Lo, J. F., and Silver, S. (1999) Nat. Med. 5, 183–188
18. Bensing, C., Mitra, B., and Rosen, B. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14326–14331
19. Rutherford, J. C., Cavet, J. S., and Robinson, N. (1999) J. Biol. Chem. 274, 25827–25832
20. Axelsen, K. B., and Palmgren, M. G. (2001) Plant Physiol. 126, 696–706
21. Bensing, C., Sun, Y., Mitra, B., and Rosen, B. P. (1998) J. Biol. Chem. 273, 32614–32617
22. Sharma, R., Rensing, C., Rosen, B. P., and Mitra, B. (2000) J. Biol. Chem. 275, 3873–3878
23. Ocker, J., and Haltia, T. (1999) Biochemistry 38, 14109–14116
24. Mitra, B., and Sharma, R. (2001) Biochemistry 40, 7694–7699
25. Fairbanks, G., and Avruch, J. (1972) J. Supramol. Struct. 1, 66–75
26. Chan, K. M., Delfert, D., and Munger, K. D. (1986) Anal. Biochem. 157, 375–380
27. Hou, Z. J., Narindrasorasak, S., Bhushan, B., Sarkar, B., and Mitra, B. (2001) J. Biol. Chem. 276, 40858–40863
28. Tsai, K. J., and Linet, A. L. (1993) Arch. Biochem. Biophys. 305, 267–270
29. Wunderh-Ye, H., and Soliz, M. (2001) Biochem. Biophys. Res. Commun. 280, 713–719
30. Soliz, M., and Camakaris, J. (1997) FEBS Lett. 412, 165–168
31. Tsivkovskii, R., Eisses, J. F., Kaplan, J. H., and Lutsenko, S. (2002) J. Biol. Chem. 277, 976–983
32. Buch-Pedersen, M. J., Venema, K., Serrano, R., and Palmgren, M. G. (2000) J. Biol. Chem. 275, 39167–39173
33. East, L., Bertini, I., Coss-Baffoni, S., Finney, L. A., Outten, C. E., and O’Halloran, T. V. (2002) J. Mol. Biol. 323, 883–897
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*J. Biol. Chem. 2003, 278:28455-28461.*
doi: 10.1074/jbc.M301415200 originally published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301415200

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