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Metal Selectivity of a Cd-, Co-, and Zn-Transporting P_{1B}-type ATPase

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Abstract

The P_{1B}-ATPases, a family of transmembrane metal transporters important for transition metal homeostasis in all organisms, are subdivided into classes based on sequence conservation and metal specificity. The multifunctional P_{1B-4}-ATPase CzcP is part of the cobalt, zinc, and cadmium resistance system from the metal-tolerant, model organism *Cupriavidus metallidurans*. Previous work revealed the presence of an unusual soluble metal-binding domain (MBD) at the CzcP N-terminus, but the nature, extent, and selectivity of the transmembrane metal-binding site (MBS) of CzcP have not been resolved. Using homology modeling, we show that four wholly conserved amino acids from the transmembrane (TM) domain (Met²⁵⁴, Ser⁴⁷⁴, Cys⁴⁷⁶, and His⁸⁰⁷) are logical candidates for the TM MBS, which may communicate with the MBD via interactions with the first TM helix. Metal-binding analyses indicate that wild-type (WT) CzcP has three MBSs, and data on N-terminally truncated (MBD) CzcP suggest the presence of a single TM MBS. Electronic absorption and electron paramagnetic resonance spectroscopic analyses of MBD CzcP and variant proteins thereof provide insight into the details of Co²⁺ coordination by the TM MBS. These spectroscopic data, combined with in vitro functional studies of WT and variant CzcP proteins, show that the side chains of Met²⁵⁴, Cys⁴⁷⁶, and His⁸⁰⁷ contribute to Cd²⁺, Co²⁺, and Zn²⁺ binding and transport, whereas the side chain of Ser⁴⁷⁴ appears to play a minimal role. By comparison to other P_{1B-4}-ATPases, we suggest that an evolutionarily adapted flexibility in the TM region likely afforded CzcP the ability to transport Cd²⁺ and Zn²⁺ in addition to Co²⁺.

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Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://doi.org/10.1021/acs.biochem.6b01022) at DOI: 10.1021/acs.biochem.6b01022. Distinctive features of the CzcP homology model (Figure S1). Spatial proximity of the MBD MBS1 and MBS2 to the conserved TM residues in the CzcP homology model (Figure S2). Partial sequence alignment showing conservation of CzcP TM residues among several other biochemically characterized P_{1B}-ATPases (Figure S3). SDS-PAGE analysis of CzcP proteins (Figure S4). Absorbance difference spectrum of Co²⁺-loaded S474A/C476A CzcP (Figure S5) (PDF)

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Notes

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The P_{1B} -ATPases, members of the larger P-type ATPase superfamily, are integral membrane proteins that utilize energy derived from ATP hydrolysis to transport metal ions across lipid bilayers.^{1,2} The average P_{1B} -ATPase comprises three domains^{2,3} (Figure 1A): a soluble ATP-binding domain (ATPBD) that houses nucleotide-binding (N) and phosphorylation (P) subdomains, a soluble actuator domain (AD) that aids in dephosphorylation, and a transmembrane helical (TM) region that provides the cation translocation pathway. Additional soluble metal-binding domains (MBDs) may be found at the N- and/or C-termini, but the exact function of these domains remains unclear.⁴ The P_{1B} -ATPases are found in all kingdoms of life and are divided into seven subfamilies (P_{1B-1} to P_{1B-7}) based on sequence homology, the presence/absence of conserved MBDs, and metal substrate specificity.^{4,5} The P_{1B} -ATPase catalytic cycle is believed to follow an adapted form of the classical Post-Albers cycle (Figure 1B),^{6,7} which has been studied biochemically^{8,9} and probed structurally for other P-type ATPases such as the sarcoplasmic/endoplasmic reticulum Ca^{2+} transporter (SERCA),¹⁰ the H^{+} -ATPase,¹¹ and the Na^{+}/K^{+} -ATPase.^{12,13} Several Cu^{+} - and Zn^{2+} -transporting P_{1B} -ATPases have been biochemically characterized,³ and some limited structural data exist, including crystal structures of the Cu^{+} transporter *Legionella pneumophila* CopA (*LpCopA*)¹⁴ and the Zn^{2+} transporter *Shigella sonnei* ZntA (*SsZntA*),¹⁵ both determined in the absence of metal. However, the vast majority of other P_{1B} -ATPases remain poorly understood despite their ubiquity in nature.⁴

The P_{1B} -ATPases are indispensable for metal homeostasis in nearly every living organism. In most metazoa, the loss or malfunction of P_{1B} -ATPases can be highly deleterious. Specifically, mutations in genes that encode P_{1B} -ATPases cause human diseases such as Menkes syndrome and Wilson disease,^{16–18} as well as growth and developmental abnormalities in plants and bacteria.^{19–21} The P_{1B} -ATPases may also represent an untapped frontier in combating bacterial infection and mitigating heavy metal pollution. Because some P_{1B} -ATPases are necessary for bacterial infection,^{22,23} they represent a potential target for the development of novel antibiotics. Furthermore, recent research efforts have focused on understanding how to manipulate P_{1B} -ATPases in plants and virulent bacteria to increase the accumulation of toxic thiophilic metals such as Cd, Pb, Ag, and Hg from contaminated environments.^{24,25} The lack of structural and biochemical data on most P_{1B} -ATPases has hampered development of these new technologies.

One organism with the potential to be genetically manipulated for the purposes of environmental remediation is the nonpathogenic, metal-tolerant β -proteobacterium

Cupriavidus metallidurans (formerly *Alcaligenes eutrophus* and *Ralstonia metallidurans*).^{26,27} The extremely robust and versatile *C. metallidurans* is capable of growing in a variety of metal-contaminated environments, is able to degrade exogenous xenobiotics,²⁷ can precipitate gold from contaminated soils,²⁸ and has even been shown to remove mercury from contaminated waters.²⁹ The ability of *C. metallidurans* to survive in such metal-polluted conditions derives from the plethora of metal-resistance proteins that are encoded chromosomally or on two endogenous megaplasms, pMOL28 and pMOL30.^{30,31} In addition to several resistance-nodulation-division (RND)-driven and cation diffusion facilitator (CDF) efflux systems, *C. metallidurans* possesses genes for eight P_{1B}-ATPases.^{26,31} Four are P_{1B-1}-ATPases likely responsible for Cu⁺ transport, three are P_{1B-2}-ATPases likely responsible for Zn²⁺/Cd²⁺/Pb²⁺ transport,^{31–33} and the final is a distinct P_{1B-4}-ATPase that is part of the larger gene cluster *czc* (cobalt, zinc, and cadmium resistance system) and is known as CzcP.^{26,31}

Recently we structurally and spectroscopically characterized the N-terminal MBD of the *C. metallidurans* CzcP P_{1B-4}-ATPase.³⁴ This unique domain consists of a fused ferredoxin-like motif and houses two distinct metal-binding sites (MBSs), of which one is solvent-exposed (MBS 1) and the other is located at the domain–domain interface (MBS 2). The MBD is not essential for CzcP-mediated Cd²⁺-, Co²⁺-, and Zn²⁺-stimulated ATP hydrolysis, but is required for maximal activity and for heightened Cd²⁺ resistance in *Escherichia coli*. Our combined data suggest distinct functional roles for each MBS, with one site (MBS 1) functioning in metal sequestration and delivery and the other site (MBS 2) functioning as a metallosensor. In an effort to understand how CzcP can bind and transport Cd²⁺ and Zn²⁺ in addition to Co²⁺, we have now investigated metal binding by its TM region guided by homology modeling. Using site-directed mutagenesis, ATPase assays, and electron paramagnetic resonance (EPR) spectroscopy, we show that the side chains of three key residues, Met²⁵⁴, Cys⁴⁷⁶, and His⁸⁰⁷, are functionally important for CzcP-mediated metal transport. In addition, we demonstrate that binding of Cd²⁺ within the TM region can labilize metal in the MBD, suggesting cross-talk between the two domains.

MATERIALS AND METHODS

Materials

All materials used for buffer and solution preparations were purchased from Sigma-Aldrich, RPI, and/or VWR and used as received. Detergents were purchased from Anatrace, stored at –20 °C, and used as received.

Cloning and Expression of WT CzcP and Variant Proteins

Cloning of the wildtype CzcP (WT CzcP, residues 1–829) and CzcP lacking the MBD (MBD CzcP, residues 183–829) into the pET-21a(+) (EMD Millipore) expression plasmid, which encodes for a C-terminal (His)₆ tag, was described previously.³⁴ For cleavage of the C-terminal tag, a TEV protease cleavage site (AGENLYFQSAG) was synthetically added to the CzcP gene construct prior to the plasmid-encoded (His)₆ site. Mutations M254A, S474A, C476A, S474A/C476A, and H807A were introduced using the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent). WT or MBD CzcP containing the

TEV protease cleavage site in pET-21a(+) served as the template for mutagenesis, and the following primers were used: 5'-CGCCTGCTGCGGCAACTAACGCCAGTGTATCAATTTCAAATTT-3' and 5'-AAATTTGAAATTGATACACTGGCGTTAGTTGCCGAGCAGGCG-3' (M254A); 5'-CAGGGCACACGGGGCGGCTGCAACCAGC-3' and 5'-GCTGGTTGCAGCCGCCCCGTGTGCCCTG-3' (S474A); 5'-CAATTGCCAGGGCAGCCGGGCTGGCTGCAA-3' and 5'-TTGCAGCCAGCCGGGCTGCCCTGGCAATTG-3' (C476A); 5'-GCAATTGCCAGGGCAGCCGGGGCGGCTGCAACCAGCAC-3' and 5'-GTGCTGGTTGCAGCCGCCCCGGTGGCTGGCAATTGC-3' (S474A/C476A); 5'-GGGTGCTGCCTTCAGCCATTGCAACGGCCG-3' and 5'-CGGCCGTTGCAATGGCTGAAGGCAGCACCC-3' (H807A). The presence of each correct mutation was verified by automated DNA sequencing (ACGT Inc.).

Expression of each construct was performed as described previously.³⁴ Briefly, 12 L of Luria Broth (LB) containing 100 µg/mL ampicillin and *E. coli* BL21(DE3) cells (EMD Millipore) bearing the appropriate expression plasmid were cultured at 37 °C with shaking at 200 rpm in baffled, sterile flasks until the optical density at 600 nm (OD₆₀₀) reached ~0.6–0.8. Cells were then cold shocked for 2 h at 4 °C. Afterward, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were then incubated at 18 °C, 200 rpm shaking for ~18 h, after which cells were harvested by centrifugation for 10 min at 4800g, resuspended in resuspension buffer (25 mM Tris, pH 7.5, 100 mM sucrose, and 10 mM EDTA), flash-frozen in N₂(l), and stored at –80 °C until further use. For preparation of large quantities of enzyme necessary for EPR analyses, cells were grown in ZYM-5052 autoinduction media³⁵ at 37 °C with shaking at 200 rpm. Once the OD₆₀₀ reached ~0.5, the temperature was lowered to 18 °C, shaking was continued, and cells were harvested after ~22 h, flash-frozen in N₂(l), and stored at –80 °C until further use.

Purification of WT CzcP and all variant proteins was performed as described previously,³⁴ with all steps occurring at 4 °C unless otherwise noted. Briefly, cells were thawed and homogenized via stirring. Solid phenylmethylsulfonyl fluoride (PMSF, Pierce; 50–100 mg) and solid deoxyribonuclease I (Bovine, Sigma-Aldrich; 50–100 mg) were both added immediately before lysing the cell solution by multiple passes through a cooled 15 000-p.s.i. microfluidizer. Cellular debris was pelleted via centrifugation for 45 min at 8000g, the remaining supernatant was decanted, and membranes were pelleted by ultracentrifugation for 1 h at 163000g. Membranes were then washed and homogenized in fresh resuspension buffer before being pelleted again by ultracentrifugation for 45 min at 163000g. Washed and pelleted membranes were then resuspended in resuspension buffer in which the EDTA had been omitted. The concentration of protein in these membranes was determined using the detergent-compatible (DC) Lowry assay (Bio-Rad). Protein was solubilized by the addition of a 10% (w/v) stock *n*-dodecyl-β-D-maltopyranoside (DDM) to ~1% (w/v) final detergent concentration and ~3 mg/mL final protein concentration with vigorous stirring for ~2 h. The solution was clarified by ultracentrifugation for 1 h at 163000g. The supernatant was then applied to two tandem 5 mL HiTrap Chelating HP columns (GE Healthcare) that had been charged with Ni²⁺ and equilibrated with 8 column volumes of buffer A (25 mM Tris, pH 8.0,

100 mM sucrose, 500 mM NaCl, and 0.05% (w/v) DDM) with an additional 21 mM imidazole. The column was then washed with 12 column volumes of buffer A with an additional 50 mM imidazole. Proteins were eluted by washing with buffer A containing an additional 150 mM imidazole. Fractions were concentrated using a 15 mL Amicon 100 kDa MWCO spin concentrator (Millipore) and buffer exchanged utilizing a PD-10 column (BioRad) into 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM TCEP, 0.02% (w/v) DDM. Protein concentration was determined using the DC Lowry assay, and purity was assessed via 15% SDS-PAGE analysis.

To remove the C-terminal (His)₆ tag, all proteins were incubated with TEV protease. Briefly, enzyme and protease were mixed in a ~ 3:1 (v/v) ratio in buffer composed of 50 mM Tris, pH 7.5, 1 mM DTT, 0.5 mM EDTA, and 0.02% (w/v) DDM. This mixture was incubated overnight at 4 °C for 12–16 h, after which excess DTT was removed using a PD-10 desalting column (GE Healthcare) via buffer exchange into 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM TCEP and 0.02% (w/v) DDM. The exchanged protein was then incubated with a 1 mL HisPur Ni-NTA spin column (Agilent) for ~1 h at 4 °C. Cleaved protein was washed off of the column into 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM TCEP and 0.02% (w/v) DDM and concentrated using a 100 kDa MWCO spin concentrator (Millipore). Purity was assessed via 15% SDS-PAGE analysis, and cleavage was verified via Western blotting and subsequent immunostaining with an anti-(His)₆ antibody (Sigma-Aldrich).

CzcP Homology Modeling

The CzcP homology model was generated using the Robetta Full-Chain Protein Structure Prediction Server (<http://rosetta.bakerlab.org/>).^{36,37} The full-length CzcP amino acid sequence (829 residues) was used for input. The core ATPase structure of CzcP was modeled based on *LpCopA* (PDB ID 3RFU, chain A, in the presence of K⁺, Mg²⁺, and AlF₄⁻), and the apo CzcP MBD was modeled based on the structure of the two apo tandem ferredoxin-like motifs from *Bacillus subtilis* CopA (PDB ID 1P6T). The final CzcP model consists of the two assembled domains and comprises residues 1–829 of the native amino acid sequence. Conservation of sequence identities and similarities was calculated using the Mobyle online server (<http://mobyle.pasteur.fr/cgibin/portal.py>) maintained by the Pasteur Institute.³⁸

Metal Loading and Analysis

Metal-loading analyses were performed on CzcP constructs in the final exchange buffer (vide supra) with an additional 400 mM NaCl and 20% (v/v) glycerol. Complete loading of Cd²⁺, Co²⁺, and Zn²⁺ was achieved via slow addition of 10 mol equiv of the buffered metal cation solution at 4 °C to the apo protein. After complete addition of metal, the solution was rocked gently overnight (typically 12–16 h) at 4 °C. Partial loading of Co²⁺ for EPR analyses was achieved via slow addition of 0.75 mol equiv of the buffered metal cation solution at 4 °C to the apo protein. After complete addition of metal, the solution was rocked gently for 1 h at 4 °C. Excess metal was removed via buffer exchange on a PD-10 column into 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM TCEP and 0.02% (w/v) DDM. Final protein concentrations were determined using the DC Lowry assay. For metal analysis, protein was digested in 5 mL of 3% TraceSelect HNO₃ (Sigma-Aldrich). Standards containing Cd²⁺,

Co²⁺, and Zn²⁺ were similarly prepared in 3% TraceSelect HNO₃. Metal content was measured using a Thermo-Fisher iCAP 7600 inductively coupled plasma optical emission spectrometer (ICP-OES, Quantitative Bioelement Imaging Center, Northwestern University). Data were interpolated from linear curves generated using standard solutions of known concentration and are the average of at least three independent samples. Metal-binding data for the CzcP MBD were obtained from a prior publication³⁴ and are included for sake of comparison.

Electronic Absorption and EPR Spectroscopies

All electronic absorption spectra were collected at room temperature using an Agilent 8453 UV–vis spectrophotometer set to a spectral bandwidth of 1 nm. Protein (typically ~25–50 μ M) was measured in a 1 cm path length quartz cuvette.

Samples for EPR analyses were concentrated to 0.2–0.7 mM protein in 25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM TCEP, and 0.02% (w/v) DDM, with 0.75:1 (substoichiometrically loaded) Co/protein ratios. A small scoop of solid sodium dithionite crystals was added prior to sample analysis to ensure all Co ions were in the divalent state and to eliminate EPR signals from contaminating ferrihemoproteins by reduction to the ferrous state. Solutions were aliquoted into custom quartz Q-band tubes, frozen in N₂(l), and stored until analyzed. All spectra shown were collected using a continuous-wave (CW) Q-band (35 GHz) spectrometer with dispersion mode detection under rapid passage conditions^{39,40} at 1.6 K. Additional measurements were collected on a CW Q-band Bruker EMX spectrometer utilizing an Oxford Instruments CF935 cryostat at ~18 K in absorption mode detection.

ATPase Activity Assays

ATPase activity assays were performed as described previously³⁴ using the malachite green assay.⁴¹ Briefly, stocks of CzcP proteins were diluted to 0.05–0.10 mg/mL using stock buffer with a final composition of 100 mM Tris, pH 7.5, 3 mM MgCl₂, 100 mM NaCl, 10 mM TCEP, 0.1 mg/mL asolectin, and 0.01% (w/v) DDM. For metal stimulation assays, stock solutions of the metal salt (CdSO₄, CoCl₂, and ZnCl₂) were added to a final concentration of 50 μ M. Stock solutions of substrate (Na₂ATP, 1.5 mM final concentration) were added to initiate the reaction; assays were carried out in 1.5 mL Eppendorf tubes at 30 °C with 300 rpm shaking for 10–15 min, depending on the construct. The reaction was then immediately halted by addition of a 3:1 (v/v) ratio of working solution (1.05% (w/v) ammonium molybdate tetrahydrate, 0.0338% (w/v) malachite green carbinol, 1.0 M HCl) to the protein assay solution. After addition of 100 μ L of 34% (w/v) sodium citrate, the absorbance at 660 nm of the enzyme-containing solution was measured using an Agilent 8453 spectrophotometer, and the concentration of inorganic phosphate [P_i] was interpolated using a standard curve. Specific activity data are corrected against background hydrolysis of Na₂ATP in the absence of enzyme.

RESULTS AND DISCUSSION

The CzcP Homology Model

While no structure of the full-length P_{1B-4}-ATPase CzcP exists, we were able to generate a homology model from the crystal structure of the P_{1B-1}-ATPase LpCopA (PDB ID 3RFU; AlF₄⁻, pseudo E₂P_i state) (Figure 2).¹⁴ Interestingly, CzcP and LpCopA share both higher sequence identity (~36%) and sequence similarity (~56%) than CzcP and SsZntA (~34% and ~54%, respectively), despite the fact that CzcP and SsZntA transport somewhat similar metal substrates. The homology modeling process recognized two separate domains, the core P_{1B}-ATPase region and the N-terminal MBD, and modeled the two separately before combining them into a final model. To prevent biasing the model toward the structure of the Cd²⁺-bound CzcP MBD, we used the solution structure of the apo tandem ferredoxin-like domains from *B. subtilis* CopA as our starting input for the N-terminal MBD in CzcP.⁴² Remarkably, the predicted structure of the apo CzcP MBD bears a strong resemblance to that of the novel ferredoxin-like fold fusion found in the Cd²⁺-bound CzcP MBD structure (rmsd ~0.5 Å over 146 Cα atoms, Figure S1A), suggesting that our final composite model may be a good structural representation of CzcP.

In contrast to several P_{1B-4}-ATPases that are predicted to contain six TM helices,⁴ the model includes eight TM helices for full-length CzcP. In accordance with previous naming conventions for the Cu⁺- and Zn²⁺-transporting P_{1B}-ATPases,⁴ the first TM helices of CzcP are labeled “A” and “B”. Interestingly, the model also includes a region of approximately 40 amino acids at the C-terminus of the MBD. These residues were disordered in the structure of the Cd²⁺-loaded MBD,³⁴ and in this model, they form a helix followed by an extended loop region, which appears to bow TM helix A (Figure 2A, Figure S1B). This bowing of TM helix A may facilitate communication between the TM region, the MBD, and the AD. However, the two MBSs of the MBD appear to be ~30–35 Å from residues that constitute the TM MBS (vide infra). The homology model suggests that the first metal-binding, ferredoxin-like domain of the CzcP MBD may be rotated slightly toward the TM region (Figure S2). We have previously demonstrated that the solvent-exposed MBS of this domain is important for metal transport, but this model does not reveal a clear path from this MBS location to the TM region. Thus, it remains unclear whether metal can be delivered from the MBD to the TM MBS.

Using this model, which comprises the entire CzcP polypeptide, as a structural guide, we identified four potential TM metal-binding residues that might be involved in the cation translocation process. These residues, of which the side chains are within 3–8 Å of one another, are Met²⁵⁴, Ser⁴⁷⁴, Cys⁴⁷⁶, and His⁸⁰⁷ (Figure 2B). The latter three residues comprise the diagnostic SPC motif in TM helix 4 and the onset of the HEGT motif in TM helix 6.⁴ To our knowledge, this model represents the first indication that a Met residue in a P_{1B-4}-ATPase may be involved in metal translocation. We examined several hundred P_{1B-4}-ATPase sequences and found the analogous residues to be conserved in all sequences, regardless of the presence or absence of a MBD. Partial alignments of the CzcP sequence with those of other biochemically characterized P_{1B-4}-ATPases (Figure 3) indicate that these conserved residues likely occupy similar locations in the TM region and may be functionally

relevant. Additionally, we aligned these sequences with those of several biochemically characterized P_{1B-1}-, P_{1B-2}-, and P_{1B-3}-ATPases (Figure S3). While Met²⁵⁴ does not appear to be analogous to the “entryway” Met that is conserved in the Cu⁺-transporting P_{1B-1}-ATPases,⁴³ there is a Met residue found in an analogous position to Met²⁵⁴ in the Zn²⁺/Cd²⁺/Pb²⁺-transporting P_{1B-2}-ATPases. It is possible that utilization of this Met residue, in addition to several conserved residues in TM helices 4 (SPC motif) and 6 (HEGT motif) (Figure 3), may be important for CzcP to mediate the transport its diverse, thiophilic metal substrates (Cd²⁺, Co²⁺, and Zn²⁺).

Metal Binding to WT CzcP

To assess the number of potential MBSs in CzcP, we tested the ability of the WT enzyme to bind and retain its metal substrates. Upon overnight incubation of WT CzcP with a 10-fold excess of Co²⁺ or Zn²⁺ and subsequent desalting, we found that ~3 mol equiv of metal were retained (Table 1). The electronic absorption spectrum of Co²⁺-loaded WT CzcP normalized per mole of metal (Figure 4) displays weak transitions in the 450–700 nm region ($\epsilon \approx 100$ –200 M⁻¹cm⁻¹), most consistent with tetrahedral, high-spin Co²⁺.^{44–46} A strong transition at ~310 nm is also observed and is likely attributable to Cys(thiolate) to Co²⁺ ligand-to-metal charge-transfer (LMCT). Literature values for the magnitude of the molar absorptivity of this transition vary, but a similar value to those previously reported^{45,47} ($\epsilon \approx 2000$ –4000 M⁻¹ cm⁻¹ per Co²⁺ ion) is only achieved when normalized against three MBSs, suggesting that all three Co²⁺-loaded sites utilize a Cys(thiolate) in their ligation sphere. These results are consistent with our previous studies that revealed Cys(thiolate) ligation in two tetrahedral N-terminal MBSs³⁴ and suggest that a Cys(thiolate) ligand binds to a tetrahedral Co²⁺ ion in the TM region as well.

Surprisingly, overnight incubation of WT CzcP with Cd²⁺ and subsequent desalting resulted in the binding of only 1.10 mol equiv of metal (Table 1). This result is not a function of the inability to bind metal in the remaining two MBSs, as incubation of the Cd²⁺-bound species with Zn²⁺ and subsequent desalting resulted in ~3 mol equiv of metal bound (0.79 mol equiv Cd²⁺ and 2.16 mol equiv Zn²⁺, Table 1). However, addition of Co²⁺ to Cd²⁺-bound WT CzcP resulted in minimal (0.35 mol equiv) additional metal loading (Table 1). A possible explanation for these findings is that binding of Cd²⁺, but not Zn²⁺ and Co²⁺, to WT CzcP in the TM region affects binding of Cd²⁺ and Co²⁺ by the remaining two MBSs in the MBD. To test whether binding of Cd²⁺ in the TM region affects binding of metal in the MBD, we generated and purified a variant protein in which residues Ser⁴⁷⁴ and Cys⁴⁷⁶ of the SPC motif are mutated to Ala to generate an APA TM helix 4 motif (S474A/C476A CzcP, Figure S4). Consistent with our hypothesis, S474A/C476A CzcP is fully capable of retaining ~2 mol equiv of Cd²⁺. Likewise, this variant binds ~2 mol equiv of either Co²⁺ or Zn²⁺, and the electronic absorption spectrum of the Co²⁺-loaded sample strongly resembles that of the Co²⁺-loaded CzcP MBD (Figure S5), suggesting that the 2 mol equiv of metal are bound in the MBD of S474A/C476A CzcP and that Ser⁴⁷⁴ and/or Cys⁴⁷⁶ are indeed ligands to the TM site.

This result could explain the high rate of Cd²⁺-stimulated ATP hydrolysis compared to Co²⁺- and Zn²⁺-stimulated ATP hydrolysis.³⁴ Co²⁺ and Zn²⁺ are generally exchange labile,

whereas Cd^{2+} is generally exchange inert. It is therefore plausible that Cd^{2+} may require a priming mechanism for its transport, especially if metal is delivered to the TM region from the MBD. Binding of Cd^{2+} in the TM region might elicit dynamical alterations in the geometry of the MBD MBSs, which could prepare metal in this domain to be delivered to the TM site immediately following metal release from the enzyme in the E_2 state and return of the enzyme to the E_1 state. Metal binding in the TM region may be propagated along the bowed TM helix A to the second ferredoxin-like domain (Figure S1), which we previously observed in our Cd^{2+} -bound crystal structure to have higher thermal motion than the first ferredoxin-like domain.³⁴ Because MBD MBS2 both lies at the interface of the two ferredoxin-like domains (Figures S1 and S2), and is connected via a short loop and H-bonding to the first ferredoxin-like domain that houses MBD MBS1, it is plausible that binding in the TM region may be communicated throughout the entire soluble N-terminal region. Such changes in the MBD MBSs could lead to Cd^{2+} or Co^{2+} loss from this domain during buffer exchange and manipulation prior to metal analysis. In contrast, our prior spectroscopic measurements indicate that Zn^{2+} in the MBD might adopt a higher coordination number,³⁴ which could explain why Cd^{2+} -bound CzcP will bind additional Zn^{2+} but not additional Cd^{2+} or Co^{2+} . These results combined with our prior studies suggest that there is cross-talk between the TM region and the MBD of CzcP, which would be necessary if the MBD plays a regulatory role in metal transport.

The CzcP Transmembrane Metal-Binding Site

To characterize the TM MBS without interference from the MBD sites, we purified a truncated version of CzcP in which the N-terminal MBD was removed (MBD CzcP, Figure S4). Overnight incubation of the C-terminal (His)₆ tag cleaved MBD CzcP with Cd^{2+} , Co^{2+} , or Zn^{2+} , and subsequent desalting resulted in the retention of ~1 mol equiv of each metal, suggesting the presence of one TM MBS (Table 1). The variant protein H807A

MBD CzcP (Figure S4) retained the ability to bind ~1 mol equiv of all metal ions, whereas S474A and C476A MBD CzcP bound 0.45 and 0.61 mol equiv of Co^{2+} , respectively, and M254A MBD CzcP bound 0.34 and 0.26 mol equiv of Cd^{2+} and Co^{2+} , respectively, indicating diminished metal-binding capacity in the Ser, Cys, and Met variants (Table 1).

Consistent with the WT CzcP data, the electronic absorption spectrum of “singly-loaded” Co^{2+} -loaded MBD CzcP displayed weak transitions in the 450–700 nm region ($\epsilon \approx 100\text{--}200 \text{ M}^{-1} \text{ cm}^{-1}$) as well as a strong transition at ~305 nm that may be attributed to a Cys(thiolate) to Co^{2+} LMCT (Figure 4). The spectrum of Co^{2+} -loaded C476A MBD CzcP lacks this strong LMCT transition at ~305 nm and exhibits diminished and shifted d → d ligand-field transitions in the visible region ($\epsilon < 100 \text{ M}^{-1} \text{ cm}^{-1}$). This result strongly suggests that Cys⁴⁷⁶ binds directly to the Co^{2+} ion in the TM MBS, although we cannot rule out the possibility that loss of H-bonding between the cysteine side chain and solvent or another amino acid side chain diminishes the LMCT transition.

To gain greater insight into the nature of the TM MBS, we characterized Co^{2+} -bound MBD CzcP and MBD CzcP TM MBS variants using EPR spectroscopy. To target the high-affinity MBS and to prevent any additional metal binding we loaded the proteins substoichiometrically with Co^{2+} . The ~2K continuous-wave (CW) Q-band EPR

measurement yields the absorption-display spectra of Figure 5, rather than the more familiar derivative-display spectra of conventional spectrometers. For reasons that will become clear immediately, we first discuss the MBD CzcP and H807A variant, and then turn to the S474A and C476A variants.

The low-field edge of the Co^{2+} -bound MBD CzcP spectra corresponds to the $g_1 = 5.87$ (Figure 5D) feature of a $S = 3/2$ Co^{2+} ion with a highly rhombic zero-field splitting (ZFS) interaction.⁴⁸ The dip in intensity as the field is raised from the low-field edge of the spectrum is an artifact associated with enhanced spin relaxation at fields away from g_1 . Although the additional features expected in the spectrum for such a spin system are not resolved in this spectrum, a feature at $g_2 \approx 2.8$ is observed at slightly higher temperature (data not shown). A signal with these two g -values is well explained by equations⁴⁸ for the lower Kramers doublet of a high spin, $S = 3/2$ Co^{2+} with an axial ZFS parameter (defined as the energy difference between Kramers doublets) $D > 0$, D substantially larger than the microwave quantum, $D \gg h\nu$, ZFS rhombicity parameter, $\lambda = E/D \approx 0.25$, and with intrinsic g -values of $g_x, g_y \approx 2.25$. The predicted feature $g_3 \ll 2$ could not be resolved. Interestingly, the MBD CzcP spectrum is different from the response exhibited by $S = 3/2$ Co^{2+} bound to sCoaT, the spectrum of which is also described as $D > 0$, $D \gg h\nu$, but with $\lambda \approx 0$ and intrinsic g -values of $g_x, g_y \approx 2.0$.⁴⁹

To identify ligands involved in Co^{2+} binding at the TM MBS, we examined the substoichiometrically loaded MBD CzcP variants. The M254A variant does not stably bind Co^{2+} (vide supra, Table 1). The H807A variant does stably bind Co^{2+} , but the mutation abolishes the signal of Co^{2+} in the native TM MBS of MBD CzcP and instead produces a broad Co^{2+} EPR response with signal with $g_{\perp} \approx 4.0$ (Figure 5A), similar to that observed in sCoaT.⁴⁹ Careful inspection of the spectrum indicates that it actually arises from more than one such Co^{2+} species. Thus, the H807A mutation has eliminated Co^{2+} binding at the native TM MBS, as would be predicted for His⁸⁰⁷ as a TM MBS ligand, with the new Co^{2+} signal associated with Co^{2+} binding elsewhere. We note that the much greater intensity of the signal from the secondary sites compared to that of the native TM MBS (for comparable Co^{2+} concentrations) is a function of a number of differences between the characteristics of the two spin systems,⁵⁰ and not of higher occupancy of the secondary Co^{2+} sites. In fact, the sites which are observed for the H807A variant must be of substantially lower affinity than the native TM MBS, as they are not populated measurably in competition with the native TM MBS; even a few percent occupancy of this intense signal would be detectable in the response from MBD CzcP.

We use the signals for MBD CzcP and the H807A variant as the basis for discussing those from the S474A and C476A variants. The signal for the S474A variant (Figure 5C) shows the response associated with Co^{2+} bound at the native TM MBS of MBD CzcP, $g_1 = 5.85$, but with its intensity reduced approximately 3-fold, and also shows a very minor contribution (estimated at <1% of the total observed Co^{2+} resonant spins) from the intense broad $g_{\perp} \approx 4.0$ Co^{2+} resonance observed for the H807A variant. This indicates that binding at the TM MBS has been weakened, but not abolished by mutation of Ser⁴⁷⁴, and that this mutation also weakens the binding at the secondary site, as it now only partially takes up the unbound Co^{2+} . Thus, the side chain of Ser⁴⁷⁴ is not a ligand of the TM MBS, but Ser⁴⁷⁴ is

involved in the stabilization of Co^{2+} binding by the TM MBS and the secondary site. This observation would be consistent with a proposal in which Co^{2+} ligation involves the Ser⁴⁷⁴ main-chain carbonyl oxygen, as observed in other P-type ATPases (i.e., SERCA and Na^+/K^+ -ATPase), and that the secondary site corresponds to that where Co^{2+} binds in sCoaT.^{13,51} The hydroxyl side chain could then potentially be involved in hydrogen bonding interactions, with its loss in the S474A variant destabilizing the TM site. This interpretation is consistent with prior studies of a P_{1B}-ATPases which have not indicated direct metal ligation by a Ser hydroxyl side chain.

The C476A MBD CzcP spectrum (Figure 5B), like that of the H807A variant, is dominated by a broad $g_{\perp} \approx 4.0$ Co^{2+} resonance, indicating that this mutation, like H807A, has disrupted the native TM MBS and caused the substoichiometrically added Co^{2+} to bind at a secondary site. This strongly implies that the Cys⁴⁷⁶ side chain is a TM MBS ligand, as also inferred from the optical spectroscopy (Figure 4). Examination of the C476A spectrum reveals a subtle feature at $g \approx 6$ (Figure 5B, asterisk) indicative of Co^{2+} binding in a distinct geometry from that associated with the $g_{\perp} \approx 4.0$ Co^{2+} resonance, potentially in a similar geometry to the native TM MBS.

In summary, mutagenesis of the putative TM MBS ligands combined with EPR data provides evidence that Met²⁵⁴, Cys⁴⁷⁶, and His⁸⁰⁷ indeed contribute side chain Co^{2+} ligands at the TM MBS of CzcP. Moreover, these results demonstrate that the Ser⁴⁷⁴ side chain is not a ligand, but that Ser⁴⁷⁴ is involved in stabilizing the TM MBS, with the Ser⁴⁷⁴ main-chain carbonyl oxygen perhaps providing a Co^{2+} ligand.

Residues Met²⁵⁴, Cys⁴⁷⁶, and His⁸⁰⁷ of CzcP are Necessary for Maximal ATPase Activity

To assess the functional relevance of Met²⁵⁴, Ser⁴⁷⁴, Cys⁴⁷⁶, and His⁸⁰⁷, we compared the rates of metal-stimulated ATP hydrolysis by WT CzcP and the series of variants (M254A, S474A, C476A, S474A/C476A, and H807A). The assays were performed in the presence of 50 μM final concentration metal (Figure 6A), which were the optimal activity conditions determined previously;³⁴ under these conditions, WT CzcP hydrolyzes ATP at rates of 200 nmol P_i mg^{-1} min^{-1} ($+\text{Cd}^{2+}$), 60 nmol P_i mg^{-1} min^{-1} ($+\text{Co}^{2+}$), and 40 nmol P_i mg^{-1} min^{-1} ($+\text{Zn}^{2+}$) at 30 °C. Mutation of the wholly conserved Met²⁵⁴ dramatically reduces Cd^{2+} - and Co^{2+} -stimulated ATPase activity (~8% and ~32% activity of WT, respectively, Figure 6A), whereas Zn^{2+} -stimulated ATPase activity remained virtually unchanged compared to WT, suggesting Met²⁵⁴ is involved in Cd^{2+} and Co^{2+} transport, but not Zn^{2+} transport. With respect to the conserved SPC motif, mutation of Ser⁴⁷⁴ modestly decreases Co^{2+} -stimulated ATP hydrolysis (~66% activity of WT, Figure 6A), whereas Cd^{2+} - and Zn^{2+} -stimulated ATP hydrolysis remain virtually unchanged compared to WT. However, a more dramatic decrease is observed upon mutation of Cys⁴⁷⁶ (~4%, ~24%, and ~30% Cd^{2+} -, Co^{2+} -, and Zn^{2+} -stimulated ATP hydrolysis activity of WT, respectively), suggesting that this residue plays an important role in transport of all three metals (Figure 6A). That Cd^{2+} -stimulated ATP hydrolysis is more strongly affected by the Met²⁵⁴ and Cys⁴⁷⁶ mutations than the Ser⁴⁷⁴ mutation is consistent with Cd^{2+} being a much softer, more thiophilic Lewis acid, which likely relies more heavily on the metal-sulfur interactions than Co^{2+} and Zn^{2+} , which are borderline hard/soft Lewis acids.⁵² Unsurprisingly, a similarly strong decrease in metal-

stimulated ATP hydrolysis (~2%, ~16%, and ~20% Cd^{2+} -, Co^{2+} -, and Zn^{2+} -stimulated ATP hydrolysis activity of WT, respectively, Figure 6A) is observed for the S474A/C476A CzcP variant, in which both conserved TM 4 residues are altered. Demonstrating the importance of the sole His imidazole side chain that points into the putative TM MBS, mutation of His⁸⁰⁷ to Ala effectively abrogates Cd^{2+} -stimulated ATP hydrolysis (<1% of WT) and results in a strong decrease in Co^{2+} - (~17% of WT) and Zn^{2+} -stimulated (~16% of WT) ATP hydrolysis (Figure 6A). This stark diminution in metal-stimulated ATPase activity for all substrates tested indicates that His⁸⁰⁷ is a key ligand in the cation translocation process.

To ascertain that the observed decreases in metal-stimulated ATPase activity derive from alterations solely in the TM region and not from competition between the TM MBS and the MBD for metal binding, we performed the same ATPase assays utilizing variants of the

MBD CzcP protein. Similar trends in metal-stimulated ATPase activity are indeed observed for the MBD CzcP TM MBS variant proteins as for the WT variant proteins (Figure 6B), with the exception of the S474A/C476A MBD CzcP variant, which could not be assayed due to protein aggregation and precipitation once purified. SDS-PAGE analysis of this variant indicated comparable protein purity to other variant proteins with no observed proteolysis (Figure S4), suggesting that protein degradation was not the source of instability. It may be that the truncation of the MBD combined with altered or deleted hydrogen-bonding interactions causes destabilization or misfolding of the S474A/C476A MBD CzcP double variant that does not occur in the single variant proteins.

Metal Substrate Diversity of $\text{P}_{1\text{B-4}}$ -ATPases: A Function of the Transmembrane Metal-Binding Site?

The best biochemically and spectroscopically characterized $\text{P}_{1\text{B-4}}$ -ATPases are CzcP and sCoaT, although their substrate specificities differ dramatically, which seems to be due in part to how specific residues in TM 1, TM 4, and TM 6 of each $\text{P}_{1\text{B-4}}$ -ATPase are utilized. Whereas sCoaT employs a mixture of N/O-containing first-sphere ligands (including a putative His residue) to bind only Co^{2+} ,⁴⁹ CzcP appears to make use of N/O- and S-containing first-sphere ligands to bind multiple metal substrates despite the occurrence of both sets of metal-site residues in each transporter. This proposed coordination environment in CzcP is supported in the present study by mutations of Met²⁵⁴, Cys⁴⁷⁶, or His⁸⁰⁷ in CzcP that affect ATP hydrolysis in vitro as well as spectroscopic signatures associated with metal bound in the TM region. Surprisingly, mutation of the wholly conserved Ser⁴⁷⁴ has minimal effects on both functional and spectroscopic analyses, but metal-binding data suggest that this residue is important. It may be that the CzcP TM MBS uses the peptide carbonyl oxygen of this residue for metal binding, similar to what is observed in binding site II of SERCA⁵¹ or binding site II of the Na^+/K^+ -ATPase.¹³ This metal-binding moiety would remain conserved regardless of amino acid substitution, and hydrogen bonding at the Ser⁴⁷⁴ hydroxyl side chain could help orient the carbonyl oxygen. The more sulfur-rich composition of the TM MBS in CzcP may also be necessary to drive metal from either the MBD or a potential metallochaperone into the TM MBS. Delivery of metal from the MBD to the TM MBS would require communication between the TM region and the MBD, and we observe that binding of Cd^{2+} in the TM region appears to labilize metal in the MBD, indicating that cross-talk between these sites can occur.

Interestingly, the P_{1B-4}-ATPases have the widest metal substrate scope of any of the P_{1B} subfamilies.^{34,49,53,54} This diversity could be related to the presence or absence of an N-terminal MBD since a large percentage of P_{1B-4} sequences are predicted to bear these domains.⁴ However, the MBD cannot be the only determinant of substrate specificity, as evidenced by varying specificities of sCoaT (Co²⁺-specific and lacking an MBD),⁴⁹ MBD CzcP (Cd²⁺-, Co²⁺-, and Zn²⁺-specific),³⁴ and other P_{1B-4}-ATPases that are predicted to lack a MBD, but are capable of transporting Fe²⁺, Ni²⁺, and Zn²⁺ in addition to Co²⁺.⁵³⁻⁵⁶ Given our current and prior results on CzcP and sCoaT, it is likely that subtle differences in the TM region alter both the coordination chemistry and relative flexibility of the metal ligands to confer substrate specificity.

Remarkably, the ability to alter the selectivity of CzcP by modification of a single TM residue (e.g., Met²⁵⁴) suggests that this and/or other P_{1B}-ATPases may be genetically mutable scaffolds for imparting or changing metal transport abilities to benefit certain bacteria under environmental stress. Given that Co²⁺ is the only commonly transported metal for all biochemically characterized P_{1B-4}-ATPases, this metal may have been the first to be translocated by this subfamily. We suggest that an adaptation in an ancestral P_{1B}-ATPase that introduced flexibility in the TM MBS coordination geometry may have been the initial step toward broadening metal-substrate diversity, and each species could have further evolved to utilize this transporter based on its environmental needs and pressures. Then changes in the TM regions of some P_{1B-4}-ATPases might have allowed for transport of Cd²⁺, Zn²⁺, Fe²⁺, and Ni²⁺, of which all can accommodate multiple types of protein ligands, coordination numbers, and coordination geometries.^{57,58}

It is possible that the ability to transport a range of metal ions is only exploited under extreme environmental stress. For example, the mixed composition of the TM MBS may afford CzcP the ability to transport both borderline and soft Lewis acids. Such versatility would allow CzcP to function in concert with Zn²⁺- and Cd²⁺-transporting P_{1B-2}-ATPases in *C. metallidurans* during times of stress as well as under conditions in which Zn²⁺ or Co²⁺ transport may be CzcP's main function.²⁶ A similar scenario may occur for the Co²⁺- and Fe²⁺-transporting P_{1B-4}-ATPases. Recent studies have indicated that Fe²⁺ is transported via the P_{1B-4}-ATPases at high (mM) concentrations. Kinetic parameters indicate $K_{1/2}^{\text{Fe}}$ values at least an order of magnitude greater than that of $K_{1/2}^{\text{Co}}$ whereas the respective metal affinities are similar,⁵⁴⁻⁵⁶ suggesting that under conditions in which in vivo metal concentrations are well-controlled (buffered from the sub- μM to mM regime), Co²⁺ is a much better substrate than Fe²⁺. It may be that under circumstances in which the cell is inundated by an extreme excess of toxic or redox-active metals (e.g., Cd²⁺ or Fe²⁺), the multifunctional P_{1B-4}-ATPases (e.g., CzcP, PfeT, CtpD) are utilized to expel metal as an emergency effort to help correct metal imbalance. Thus, the P_{1B-4}-ATPases may play a unique role within the greater P_{1B}-ATPase family in ensuring bacterial survival across a wide array of environmental pressures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AD	actuator domain
ATP	adenosine-5'-triphosphate
ATPBD	ATP-binding domain
CzcP	cobalt-zinc-cadmium resistance protein
EPR	electron paramagnetic resonance
LMCT	ligand-to-metal charge transfer
MBD	metal-binding domain
MBS	metal-binding site
SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ transporter
TM	transmembrane

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Figure 1.

Architecture and mechanism of P_{1B}-ATPases. (A) Cartoon representation of a P_{1B}-4-ATPase. The average P_{1B}-4-ATPase is predicted to have 6–8 transmembrane helices (TM) (represented as blue cylinders), a soluble actuator domain (AD) between TM 2 and TM 3, and a soluble ATP-binding domain (ATPBD) between TM 4 and TM 5. Approximately half of the P_{1B}-4-ATPases are predicted to have an N-terminal metal-binding domain (MBD). Two conserved sequence motifs that are hypothesized to be important for metal binding include the Ser-Pro-Cys (SPC) motif in TM 4 and the His-Glu-Gly-Thr (HEGT) motif in TM 6. (B) Proposed post-Albers cycle for a P_{1B}-ATPase. In this scheme, the P_{1B}-ATPase cycles between a high-affinity cation-binding state (E₁) and a low-affinity cation-binding state (E₂) while being transiently phosphorylated (P) and dephosphorylated. This simplified cycle is shown for a P_{1B}-ATPase that binds and translocates any number (*n*) of divalent metal cations (M²⁺). Adapted from ref 6 and ref 7.

Figure 2.

CzcP homology model. (A) The overall model with the MBD colored gray, the AD colored purple, the ATPBD colored turquoise, and the TM region colored salmon except for the region that contains the missing residues from the CzcP MBD crystal structure and the first “bowed” TM (TM A, labeled in the left-most panel), both shown in yellow. The middle and right panels represent 90° rotations of the left and middle panels, respectively. (B) Close-up view of the boxed region of panel A, left panel, highlighting conserved residue side chains that may be important for metal binding and release in CzcP: Met²⁵⁴ (TM 1), Ser⁴⁷⁴ (TM 4), Cys⁴⁷⁶ (TM 4), and His⁸⁰⁷ (TM 6).

Figure 3.

Partial sequence alignment showing conservation of the *Cupriavidus metallidurans* CzcP (*CmCzcP*) TM residues Met²⁵⁴ (TM 1, purple), Ser⁴⁷⁴ (TM 4, orange), Cys⁴⁷⁶ (TM 4, red), and His⁸⁰⁷ (TM 6, green) in the other biochemically characterized P_{1B-4}-ATPases *Sulfitobacter* sp. NAS-14.1 CoaT (*sCoaT*), *Bacillus subtilis* PfeT (*BsPfeT*, also known as ZosA), and *Mycobacterium smegmatis* CtpD (*MsCtpD*). To the best of our knowledge, these residues are completely conserved among all known P_{1B-4} ATPase sequences.

Figure 4. Electronic absorption spectra (normalized per molar equivalent of metal) of WT CzcP + Co^{2+} (black), MBD CzcP + Co^{2+} (blue), and MBD C476A CzcP + Co^{2+} (red), plotted as molar absorptivity (ϵ ; $\text{M}^{-1}\text{cm}^{-1}$) versus wavelength (nm). Inset: close-up view of the ligand-field transitions (400 nm-700 nm).

Figure 5.

Absorption-display EPR spectra of CzcP. CW Q-band EPR spectra of H807A MBD (A), C476A MBD (B), S474A MBD (C), and Δ MBD (D) CzcP incubated with 0.75 mol equiv Co^{2+} prior to desalting and sample preparation. The sample conditions for (A) were 34.7 GHz microwave frequency, 2 G modulation amplitude, 128 ms time constant, 0.01 mW microwave power, 2 min scan time. The sample conditions for (B), (C), and (D) were the same as in (A), but with 35.1 GHz microwave frequency (B), 34.9 GHz microwave frequency (C), and 34.85 GHz microwave frequency (D). For ease of comparison due to differences in metal loading and protein concentrations, after normalizing spectra (A–D) to ratio of $[\text{Co}^{2+}]/[\text{protein}]$, spectra (A) and (B) were each scaled down by a factor of 4. Asterisk in spectrum (B) marks $g = 6.02$ from another contributing Co^{2+} species.

Figure 6.

Functional assays of CzcP and its variants. (A) Relative values for Cd²⁺-, Co²⁺-, or Zn²⁺-stimulated ATP hydrolysis by M254A, S474A, C476A, S474A/C476A, and H807A CzcP. WT activity values are normalized to 100% for Cd²⁺- (200 nmol P_i mg⁻¹ min⁻¹), Co²⁺- (60 nmol P_i mg⁻¹ min⁻¹), or Zn²⁺-stimulated ATP hydrolysis (40 nmol P_i mg⁻¹ min⁻¹). (B) Relative activity values for Cd²⁺-, Co²⁺-, or Zn²⁺-stimulated ATP hydrolysis by the MBD CzcP variants M254A, S474A, C476A, S474A/C476A, and H807A. The MBD CzcP activity values are normalized to 100% for Cd²⁺- (120 nmol P_i mg⁻¹ min⁻¹), Co²⁺- (60 nmol P_i mg⁻¹ min⁻¹), or Zn²⁺-stimulated ATP hydrolysis (20 nmol P_i mg⁻¹ min⁻¹). Data are the average ± one standard deviation of three activity measurements on the same protein preparation. N.D., not determined.

Table 1

Metal-Binding Data for Various CzcP Proteins

protein	equivalents metal bound ^d		
	Cd ²⁺	Co ²⁺	Zn ²⁺
CzcP MBD + Cd ²⁺ ^b	1.92 ± 0.47	-	-
CzcP MBD + Co ²⁺ ^b	-	2.13 ± 0.12	-
CzcP MBD + Zn ²⁺ ^b	-	-	1.98 ± 0.34
WT CzcP + Cd ²⁺	1.10 ± 0.07	-	-
WT CzcP + Co ²⁺	-	2.79 ± 0.26	-
WT CzcP + Zn ²⁺	-	-	2.53 ± 0.12
WT CzcP, Cd → Zn ^c	0.79 ± 0.46	-	2.16 ± 0.53
WT CzcP, Cd → Co ^d	0.98 ± 0.14	0.35 ± 0.10	0.20 ± 0.20 ^e
S474A/C476A CzcP + Cd ²⁺	2.37 ± 0.06	-	-
S474A/C476A CzcP + Co ²⁺	-	1.91 ± 0.17	-
S474A/C476A CzcP + Zn ²⁺	-	-	1.70 ± 0.05
MBD CzcP + Cd ²⁺	0.64 ± 0.14	-	-
MBD CzcP + Co ²⁺	-	1.18 ± 0.17	-
MBD CzcP + Zn ²⁺	-	-	1.04 ± 0.05
M254A MBD CzcP + Cd ²⁺	0.34 ± 0.10	-	-
M254A MBD CzcP + Co ²⁺	-	0.26 ± 0.14	-
M254A MBD CzcP + Zn ²⁺	-	-	0.67 ± 0.25
S474A MBD CzcP + Cd ²⁺	0.65 ± 0.02	-	-
S474A MBD CzcP + Co ²⁺	-	0.45 ± 0.02	-
S474A MBD CzcP + Zn ²⁺	-	-	1.15 ± 0.12
S474A/C476A MBD CzcP + Cd ²⁺	N.D. ^f	-	-
S474A/C476A MBD CzcP + Co ²⁺	-	N.D.	-
S474A/C476A MBD CzcP + Zn ²⁺	-	-	N.D.
C476A MBD CzcP + Cd ²⁺	1.21 ± 0.50	-	-
C476A MBD CzcP + Co ²⁺	-	0.61 ± 0.06	-
C476A MBD CzcP + Zn ²⁺	-	-	1.24 ± 0.08
H807A MBD CzcP + Cd ²⁺	1.15 ± 0.12	-	-
H807A MBD CzcP + Co ²⁺	-	0.94 ± 0.11	-
H807A MBD CzcP + Zn ²⁺	-	-	1.69 ± 0.18

^aData are reported as equivalents metal bound per equivalent of CzcP protein and are the average ± one standard deviation of three independent trials. For simplicity, “-” represents <0.1 mol equiv of metal detected after background correction of Cd²⁺, Co²⁺, and/or Zn²⁺ from buffer constituents.

^bData obtained from ref 34.

^cProtein loaded with Cd²⁺ first and then Zn²⁺.

^dProtein loaded with Cd²⁺ first and then Co²⁺.

^eThe reported Zn²⁺ value likely represents a small contaminant.

^fNot determined due to protein instability.

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