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Keywords
ATPase, Mycobacterium tuberculosis, P1B4-ATPase, iron, metal homeostasis, metal ion-protein interaction, transport metal

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Received for publication, January 29, 2016, and in revised form, March 24, 2016 Published, JBC Papers in Press, March 28, 2016, DOI 10.1074/jbc.M116.718239

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Little is known about iron efflux transporters within bacterial systems. Recently, the participation of Bacillus subtilis PfeT, a P$_{1B4}$-ATPase, in cytoplasmic Fe$^{2+}$ efflux has been proposed. We report here the distinct roles of mycobacterial P$_{1B4}$-ATPases in the homeostasis of Co$^{2+}$ and Fe$^{2+}$. Mutation of Mycobacterium smegmatis ctpJ affects the homeostasis of both ions. Alternatively, an M. tuberculosis ctpJ mutant is more sensitive to Co$^{2+}$ than Fe$^{2+}$, whereas mutation of the homologous M. tuberculosis ctpD leads to Fe$^{2+}$ sensitivity but no alterations in Co$^{2+}$ homeostasis. In vitro, the three enzymes are activated by both Fe$^{2+}$ and Co$^{2+}$ and bind 1 eq of either ion at their transport site. However, equilibrium binding affinities and activity kinetics show that M. tuberculosis CtpD has higher affinity for Fe$^{2+}$ and twice the Fe$^{2+}$-stimulated activity than the CtpJs. These parameters are paralleled by a lower activation and affinity for Co$^{2+}$. Analysis of Fe$^{2+}$ and Co$^{2+}$ binding to CtpD by x-ray absorption spectroscopy shows that both ions are five- to six-coordinate, constrained within oxygen/nitrogen environments with similar geometries. Mutagenesis studies suggest the involvement of invariant Ser, His, and Glu residues in metal coordination. Interestingly, replacement of the conserved Cys at the metal binding pocket leads to a large reduction in Fe$^{2+}$ but not Co$^{2+}$ binding affinity. We propose that CtpJ ATPases participate in the control of steady state Fe$^{2+}$ levels. CtpD, required for M. tuberculosis virulence, is a high affinity Fe$^{2+}$ transporter involved in the rapid response to iron dyshomeostasis generated upon redox stress.

Iron is an essential micronutrient required for numerous biological processes as it is used as a prosthetic group by several different enzymes (1, 2). However, in excess, it can be toxic due to its participation in Fenton chemistry and potential mismetallation in non-iron-containing metalloproteins. In this context, damage of iron-sulfur centers and mononuclear iron enzymes produced by various redox stresses are particular contributors to iron dyshomeostasis and consequent toxicity (3–6). Characterization of bacterial Fe$^{2+}$ homeostasis has mainly been focused in mechanisms of uptake (by divalent metal, siderophore, and heme transporters), transcriptional regulation (by Fur and IdeR systems), and Fe$^{2+}$ sequestration (by bacterioferritin and Dps proteins) (2, 7–9). Nevertheless, studies have suggested that cation diffusion facilitators and iron-citrate transporters participate in Fe$^{2+}$ efflux (10–12). We recently observed that Bacillus subtilis PfeT, a P$_{1B4}$-ATPase, confers Fe$^{2+}$ tolerance (13). PfeT is expressed under the control of PerR in response to peroxide exposure (14). Initial biochemical characterization showed that Fe$^{2+}$ activates isolated PfeT ATPase, leading to a higher $V_{max}$ than generated by Co$^{2+}$, which is the proposed substrate of P$_{1B4}$-ATPases (13, 15–17). Interestingly, phenotypic analysis of Listeria monocytogenes lacking the P$_{1B4}$-ATPase FrvA showed a role of this ATPase in resistance to heme toxicity (18). These observations suggest a significant role of this subfamily of P-type ATPases in Fe$^{2+}$ homeostasis (13, 14).

P$_{1B4}$-ATPases present in prokaryotes and plant chloroplasts are part of the large family of P-type ATPases (15, 19, 20). P-type ATPases are polytopic membrane proteins that transport a variety of ions using the energy provided by ATP hydrolysis (21–23). The P$_{1B}$ subgroup includes proteins responsible for the efflux of cytoplasmic transition metals including Cu$^{+}$, Zn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ (19, 22, 23). The specificity of their transport metal binding sites (TM-MBSs) is determined by invariant amino acid sequences in their last three transmembrane segments (TMs) (17, 19, 24–26). However, activation by non-cognate substrates has been reported for most P$_{1B}$-ATPase subgroups (22, 27). In particular, activation of P$_{1B4}$-ATPases by Co$^{2+}$, Ni$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ has been proposed (15–17, 28–30). We previously reported in vivo and in vitro functional studies directed at understanding the metal selectivity and consequent physiological roles of mycobacterial P$_{1B4}$-ATPases (15, 16). The presence of one or two P$_{1B4}$-ATPase-coding genes in mycobacterial species enabled comparative studies of Mycobacterium smegmatis CtpJ and Mycobacterium tuberculosis CtpJ.

*This work was supported by National Institutes of Health Grant DK066139 (to T. L. S.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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2 The abbreviations used are: TM-MBS, transmembrane metal binding site; AAS, atomic absorbance spectroscopy; EXAFS, extended x-ray absorption fine structure; LIMM, low iron defined medium; STN, streptonigrin; TM, transmembrane segment; XANES, x-ray absorption near edge spectroscopy; XAS, x-ray absorption spectroscopy; Ms, M. smegmatis; Mt, M. tuberculosis; TCEP, tris(2-carboxyethyl)phosphine.
Fe\(^{2+}\) Transport ATPases

*Mycobacterium* CtpJ and CtpD. In *vitro*, MsCtpJ and MtCtpJ display a higher activation by Co\(^{2+}\) and Ni\(^{2+}\) compared with Zn\(^{2+}\), although equilibrium binding affinities show \(K_\text{D}\) values for Zn\(^{2+}\) < Co\(^{2+}\) = Ni\(^{2+}\) (15, 16). In *vivo*, ctpJ expression is induced by Co\(^{2+}\), whereas mutant strains show accumulation and sensitivity to the metal. On the contrary, the expression of the homologous *MtctpD* is not induced by Co\(^{2+}\) but rather by redox stress. Mutation of *MtctpD* does not lead to Co\(^{2+}\) sensitivity or higher intracellular levels of this metal. Nevertheless, MtCtpD ATPase activity is partially activated by Co\(^{2+}\). Surprisingly, MtCtpD but not MtCtpJ is required for *M. tuberculosis* virulence.

Previous studies have not explored the activation of mycobacterial P\(_{1\text{B}4}\) -ATPases by Fe\(^{2+}\). Could a differential activation by Co\(^{2+}\)/Fe\(^{2+}\) explain the presence of paralogous genes in *M. tuberculosis*? Why is MtCtpD but not MtCtpJ required for virulence? To address these questions, we examined the activation of *M. smegmatis* and *M. tuberculosis* P\(_{1\text{B}4}\) ATPases by Fe\(^{2+}\) and their participation in Fe\(^{2+}\) homeostasis and stress response. In addition, we explored the molecular basis of the different Fe\(^{2+}\) and Co\(^{2+}\) ATPase activities by determining the coordination of these metals during transport by MtCtpD.

**Experimental Procedures**

*Mycobacterium* Strains and Culture Conditions—*M. smegmatis* mc2155, *M. tuberculosis* H37Rv, and derived strains were grown in 7H9 liquid medium (BD Biosciences, Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADN supplement (0.5% bovine serum albumin, 0.2% dextrose, and 0.085% NaCl) or in low iron defined medium (LIMM) containing 0.5% 1-asparagine, 0.5% KH\(_2\)PO\(_4\), 2% glycerol, 0.05% Tween 80, and 10% ADN, pH 6.8 (31). LIMM was treated with Chelex-100 (Sigma) and before use supplemented with 3.7 \(\mu\)M ZnCl\(_2\), 0.8 \(\mu\)M MnCl\(_2\), and 0.4 mM MgCl\(_2\). This medium contained less than 1 \(\mu\)M residual iron as determined by atomic absorption spectroscopy (AAS) (PerkinElmer Life Sciences PinAAcle 900z). Construction of *MsΔctpJ* (MSMEG_5403), *Mt ΔctpJ* (Rv1469), *Mt ΔctpJ* (Rv3743), and *Mt ΔctpJ:ΔctpJ* mutant and complemented strains was described previously (15, 16).

**Iron and Hemin Sensitivity Tests**—Liquid LIMM cultures of *M. smegmatis* mc2155, *M. tuberculosis* H37Rv, mutant, and complemented strains were inoculated at 0.05 \(A_{600}\) from late exponential phase cultures and supplemented with the desired concentration of FeCl\(_3\) or hemin (Sigma). A hemin stock solution (0.5% bovine serum albumin, 0.2% dextrose, and 0.085% NaCl) or in low iron defined medium (LIMM) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADN, pH 6.8 (31). LIMM was treated with Chelex-100 (Sigma) and before use supplemented with 3.7 \(\mu\)M ZnCl\(_2\), 0.8 \(\mu\)M MnCl\(_2\), and 0.4 mM MgCl\(_2\). This medium contained less than 1 \(\mu\)M residual iron as determined by atomic absorption spectroscopy (AAS) (PerkinElmer Life Sciences PinAAcle 900z). Construction of *MsΔctpJ* (MSMEG_5403), *Mt ΔctpJ* (Rv1469), *Mt ΔctpJ* (Rv3743), and *Mt ΔctpJ:ΔctpJ* mutant and complemented strains was described previously (15, 16).

**Iron and Hemin Sensitivity Tests**—Liquid LIMM cultures of *M. smegmatis* mc2155, *M. tuberculosis* H37Rv, mutant, and complemented strains were inoculated at 0.05 \(A_{600}\) from late exponential phase cultures and supplemented with the desired concentration of FeCl\(_3\) or hemin (Sigma). A hemin stock solution was prepared at 25 mg/ml in 1.4M NaOH. Cells were incubated for 16 h (*M. smegmatis*) or 5 days (*M. tuberculosis*), and \(A_{600}\) was measured. To avoid hemin interference in \(A_{600}\) readings, cells grown in hemin-containing medium were collected, washed twice with LIMM, and suspended in the original LIMM volume, and \(A_{600}\) was measured.

**Streptonigrin Sensitivity Tests**—*M. smegmatis* mc2155, *M. tuberculosis* H37Rv, mutant, and complemented strains grown in LIMM to midlog phase were diluted to 0.05 \(A_{600}\) in LIMM. The cultures were supplemented with 1 \(\mu\)g ml\(^{-1}\) streptonigrin (STN) and 10 \(\mu\)M FeCl\(_3\) as indicated in the figures. Cells were incubated for 16 h (*M. smegmatis*) or 5 days (*M. tuberculosis*), and \(A_{600}\) was measured.

**Metal Accumulation Assays**—Liquid LIMM cultures in mid-exponential phase (\(A_{600} \sim 1.0\)) were supplemented with increasing concentrations of FeCl\(_3\) and incubated for 4 (*M. smegmatis*) or 8 h (*M. tuberculosis*). After this incubation, cells were harvested and washed with 5 mM EDTA and 0.9% NaCl. Aliquots were taken for protein determinations (32). Pellets were acid-digested with 0.5 ml of NO\(_3\)H for 1 h at 80 °C and then overnight at 20 °C. Digestions were concluded by adding 1/5 volume of 30% (v/v) H\(_2\)O\(_2\) followed by a 1:5 dilution with water. Metal contents in digested samples were measured by AAS.

**Protein Expression and Purification**—Preparation of *E. coli* LMG194 Δ*copA* strains carrying *M. smegmatis* ctpJ (MSMEG_5403) or *M. tuberculosis* ctpD (Rv1469) or ctpJ (Rv3743) in pbAD-TOPO/His vectors was described previously (15, 16). Cells were grown at 37 °C in ZYP-505 autoinduction medium supplemented with 0.05% arabinose, 100 mg ml\(^{-1}\) ampicillin, and 50 mg ml\(^{-1}\) kanamycin (33). Cells were harvested at 16 h postinoculation; washed with 25 mM Tris, pH 7.0, 100 mM KCl, and 20% glycerol; and stored at −70 °C. Expressed mycobacterial proteins contained a C-terminal His\(_8\) tag sequence preceded by a tobacco etch virus protease recognition sequence. Protein purification was carried out as described previously (15, 24, 34). Briefly, cells were disrupted in a French press, and membranes were isolated by centrifugation. Membranes were treated with 0.75% dodecyl β-d-maltoside (Calbiochem), 25 mM Tris, pH 8.0, 100 mM sucrose, 500 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The solubilized membrane protein suspension was cleared by centrifugation at 163,000 \(\times\) g for 1 h, and proteins were affinity-purified using Ni\(^{2+}\)-nitrilotriacetic acid resin. The His\(_8\) tag was removed from the C terminus by treatment with His\(_8\)-tagged tobacco etch virus protease (35). Tobacco etch virus–His\(_8\) was removed by affinity purification with Ni\(^{2+}\)-nitrilotriacetic acid resin. Protein purity was analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining or Western blotting using an anti-His\(_8\) tag antibody (GenScript, Piscataway, NJ). Isolated proteins (3 mg/ml) were stored at −20 °C in 25 mM Tris, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% dodecyl β-d-maltoside, 0.01% asolectin, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Prior to ATPase activity determinations, proteins (1 mg/ml) were treated with 0.5 mM EDTA and 0.5 mM tetraethylenemamine for 45 min at room temperature. Chelators were removed using Ultra-30 Centricon (Millipore, Darmstadt, Germany) filtration devices.

**Mutagenesis of MtCtpD Metal Binding Site**—MtCtpD cloned into pBAD-TOPO/His vector was used as a template to introduce the mutations coding for the single substitutions Ser-316 (S316A), Cys-318 (C318A), His-643 (H643A), Gly-644 (G644A), Ser-645 (S645A), and Thr-646 (T646A and T646S) and the multiple replacements S316C/C318S. All mutations were introduced using a Q5\(^{®}\) site-directed mutagenesis kit (New England Biolabs, Ipswich, MA). The sequences of primers used in this study are available upon request to the corresponding author. DNA sequences were confirmed by automated sequencing.
Fe²⁺ Binding to Proteins—Metal binding to isolated enzymes was measured as described previously (15, 36). Five micromolar His-less enzyme was incubated for 1 min at 4 °C in 25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, and 1 mM TCEP. Excess metal was removed by washing in a 30-kDa-cutoff Centricon filtration device. Protein samples were acid-digested as described above, and metal concentrations were measured using AAS.

Metal binding affinities were determined using the divalent metal-binding chromophore mag-fura-2 (Invitrogen) (15, 36). Five micromolar His-less protein and 10 μM mag-fura-2 were titrated with 1 mM Fe²⁺ or Co²⁺ in Chexler-treated buffer (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, and 1 mM TCEP). Free mag-fura-2 was determined by monitoring $A_{366}$ ($\epsilon_{366}$ 29,900 1/M/cm). Free metal concentrations were calculated from $K_D = [I \cdot Me^{2+}] / [I]_{free} \cdot [Me^{2+}]_{free}$, where $I$ is mag-fura-2, $Me$ is the metal ion, and $K_D$ is the association constant of mag-fura-2 with each metal. $K_D$ values of 1.5 μM for Fe²⁺ and 2.8 μM for Co²⁺ were experimentally determined for the metal/mag-fura-2 interactions. The metal-protein $K_D$ values were calculated from $v = n[Me^{2+}]_{free}/K_D(I + ([Me^{2+}]_{free}/K_D)$ where $v$ is the molar ratio of metal bound to protein and $n$ is the apparent stoichiometry (37). Reported errors for $K_D$ and $n$ are asymptotic standard errors provided by the fitting software KaleidaGraph (Synergy, Reading, PA).

ATPase Assays—ATPase assays were performed as described (15, 24, 34). The assay mixture contained 50 mM Tris, pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 3 mM ATP, 0.01% asolectin, 0.01% dodecyl β-d-maltoside, 2.5 mM TCEP, 20 μg/ml purified protein, and freshly prepared transition metal ions at the desired concentrations. Fe³⁺ was added as FeCl₃, Cu²⁺ was added as CuSO₄, and in both cases TCEP was not included in the assay medium. Cu⁺ was obtained by including TCEP with CuSO₄ salt. Fe²⁺ and Zn²⁺ were included in the assay medium as the sulfate salts, whereas Co²⁺, Ni²⁺, and Mn²⁺ were included as their chloride salts. ATPase activity was stopped after a 20-min incubation at 37 °C, and released Pᵢ was determined (38). ATPase activity measured in the absence of transition metals was subtracted from plotted values. Curves of ATPase activity metal dependence were fit to $v = V_{max} \left[Me^{2+}\right] / \left[Me^{2+} + K_{1/2}\right]$. The reported standard errors for $V_{max}$ and $K_{1/2}$ are asymptotic standard errors reported by the fitting software KaleidaGraph.

X-ray Absorption Spectroscopy (XAS)—XAS samples were loaded in a Coy anaerobic chamber at a 1:1 metal:protein molar ratio (1.2 mM Fe²⁺ or 0.43 mM Co²⁺). Sample was injected into a Kapton-wrapped Lucite cell, flash frozen, and stored in liquid nitrogen. XAS data were collected at the Stanford Synchrotron Radiation Lightsource on beamline 7-3 equipped with a Si(220) double crystal monochromator with a harmonic rejection mirror. Fluorescence spectra were collected using a 30-element germanium solid-state detector (Canberra, Meriden, CT). During data collection, the continuous flow liquid helium cryostat (Oxford Instruments, Concord, MA) was stabilized at 10 K. Iron and cobalt data were collected using a 6-μm magnesium or a 3-μm iron filter, respectively, placed between the cryostat and the detector to reduce unassociated scattering. Iron and cobalt foil spectra were collected simultaneously with protein data for direct energy calibration of the data. The first inflection points for iron and cobalt were set at 7111.3 and 7709.5 eV, respectively. Iron XAS spectra were recorded using 5-eV steps in the pre-edge regions (6900–7094 eV), 0.25-eV steps in the edge regions (7095–7135 eV), and 0.05-Å⁻¹ increments in the extended x-ray absorption fine structure (EXAFS) region (to $k = 13.5$ Å⁻¹), integrating from 1 to 20 s in a k³-weighted manner. Cobalt XAS spectra were recorded using 5-eV steps in the pre-edge regions (7542–7702 eV), 0.25-eV steps in the edge regions (7702–7780 eV), and 0.05-Å⁻¹ increments in the extended EXAFS region (to $k = 13.5$ Å⁻¹), integrating from 1 to 25 s in a k³-weighted manner. A total of eight scans were taken for each sample, and these were then averaged.

XAS spectra were processed and analyzed using the EXAFSPAK program suite for Macintosh OSX (39). A Gaussian function was used in the pre-edge region, and a three-region cubic spline was used in the EXAFS region. EXAFS data were converted to $k$ space using $E_0$ values of 7130 and 7745 eV for iron and cobalt, respectively. Spectra were simulated using single and multiple scattering amplitude and phase functions generated using the Feff v8 software integrated within EXAFSPAK. Single scattering models were calculated for oxygen, nitrogen, and carbon to simulate possible iron- or cobalt-ligand environment. Calibrated scale factors and model $E_0$ values were not allowed to vary during fitting; the scale factor for iron was 0.95, and that for cobalt was 0.98. Iron data were fit out to a $k$ value of 13.5 Å⁻¹. Calibration from Fe²⁺ and Fe³⁺ model compounds was used for determination of $E_0$ and scale factor parameters. $E_0$ values for Fe–O and Fe–C were set at $–10$ eV. Cobalt data were fit out to a $k$ value of 13.5 Å⁻¹. Calibration from Co²⁺ model compounds was used for determination of $E_0$ and scale factor parameters. $E_0$ values for Co–O and Co–C were set at $–11.3$ eV. EXAFS spectra were simulated using both filtered and unfiltered data; however, simulation results are presented only for fits to unfiltered (raw) data. Simulation protocols and criteria for determining the best fit have been described elsewhere (25).

Results

Mycobacterial $P_{1B4}$-ATPases Confer Fe²⁺ Tolerance—We previously reported the activation of mycobacterial $P_{1B4}$-ATPases by Co²⁺ (15, 16). However, $M. tuberculosis$ CtpJ and CtpD appear to have distinct roles in Co²⁺ transport and tolerance and cellu lar response to redox stress (16). We recently observed that $B. subtilis$ PfeT, a $P_{1B4}$-ATPase in the PerR regulon, transports and confers tolerance to Fe²⁺ in addition to Co²⁺ (13). Similarly, the $L. monocytogenes$ $P_{1B4}$-ATPase, FrvA, confers resistance to heme toxicity (18). Thus, we hypothesized that selective activation by Fe²⁺ might explain the presence of the ctpJ and ctpD paralogs in the $M. tuberculosis$ genome. To test this idea, the capability of mycobacterial $P_{1B4}$-ATPases to confer tolerance to Fe²⁺ was assessed. Fig. 1 shows the growth of $M. tuberculosis$ $P_{1B4}$-ATPases in M159M supplemented with different concentrations of FeCl₃ or hemin. The $M. tuberculosis$ strain showed a growth defect at high FeCl₃ or hemin as compared with
M. smegmatis WT (Fig. 1, A and B). The complemented \( M.s\) strain, carrying the plasmid pMV306 harboring the full-length \( Msctp\) gene under the regulation of its native promoter, showed similar growth as the \( M.s\) WT. A comparable deficiency was observed in the \( M.\) strain grown in the presence of 1 mM FeCl\(_3\) (Fig. 1D). However, this mutant strain was not affected by the presence of hemin in the medium. On the contrary, mutation of the \( Msctp\) gene significantly affected the growth in both FeCl\(_3\) and hemin-supplemented medium. Interestingly, the \( M.\) double mutant strain showed a behavior identical to that of the \( M.\) strain. In all cases, complemented strains showed the growth phenotype of \( M.\) WT (Fig. 1, D and E). The data indicate that \( Msctp\) and \( Mtctp\) confer iron tolerance when cells are exposed to relatively high metal levels. Exploring their role at lower iron levels, the sensitivity to STN was tested. STN is a quinone antibiotic whose activity is correlated with intracellular iron availability (40). The \( Msctp\), \( Mtctp\), and \( Mtctp\) mutant strains displayed a significantly increased STN sensitivity in LIMM supplemented with 1 \( \mu\)g ml\(^{-1}\) STN and only 10 \( \mu\)M FeCl\(_3\) (Fig. 1C and F). In contrast to their distinct tolerance to high Fe\(^{2+}\) in the medium, there was no significant difference in the sensitivity of \( M.\) and \( M.\) strains to STN-Fe\(^{2+}\). These results suggest that to different extents mycobacterial \( P_{psa}\) ATPases contribute to Fe\(^{2+}\) homeostasis by driving this metal efflux. To further explore this hypothesis, \( P_{psa}\), \( P_{psd}\), \( P_{ps}\), and \( P_{psd}\) mutant strains were challenged with sublethal concentrations of FeCl\(_3\), and the resulting cellular Fe\(^{2+}\) levels were determined. Consistent with the iron sensitivity phenotypes (Fig. 1), Fe\(^{2+}\) accumulation was observed in mutant strains (Fig. 2, A and B). Iron levels in the \( Msctp\) strain were approximately 5 times higher than those in \( M.s\) WT (Fig. 2A). The partial recovery observed in the \( Msctp\) mutant strain complemented with \( Msctp\) appears to be associated with lower levels of transcript (35% of WT; not shown). Reinforcing the predominant role of \( Mtctp\) in Fe\(^{2+}\) homeostasis, a significant increase of Fe\(^{2+}\) content was observed in the \( Mtctp\) strain, whereas 50% smaller changes were observed in the \( Mtctp\) strain. Similar Fe\(^{2+}\) accumulation was observed in the \( Mtctp\) and \( Mtctp\) double mutant strains (Fig. 2B). These results suggest that although mycobacterial Ctp\( is involved in controlling Co\(^{2+}\) levels they also participate...
in Fe$^{2+}$ efflux, particularly when they are the only P$_{1B4}$-ATPase in the organism as in *M. smegmatis*. In contrast, MtCtpD appears to play a dominant role in maintaining the cytoplasmic Fe$^{2+}$ level in this organism.

**Distinct Biochemical Properties of Mycobacterial P$_{1B4}$-ATPase—P-ATPases couple the transmembrane transport of their substrate to ATP hydrolysis following the Albers-Post E1/E2-like mechanism (23). Consequently, the metal dependence of ATPase activity provides a starting point to analyze substrate selectivity.** Previous reports showed that MsCtpJ, MtCtpD, and MtCtpJ are differently activated by Co$^{2+}$, Ni$^{2+}$, and to a lesser extent Zn$^{2+}$ (15, 16). The activation of mycobacterial P$_{1B4}$-ATPases by Fe$^{2+}/3+$ was tested using purified proteins stabilized in lipid/detergent micelles. All three proteins were strongly activated by Fe$^{2+}$ and only minimally by Fe$^{3+}$ (Fig. 3).

For comparison, activation by Co$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ at 0.1 and 1 mM concentrations is shown. MtCtpD Fe$^{2+}$-dependent activity was ∼2-fold higher than those observed in MtCtpJ and MsCtpJ (Table 1 and Fig. 3D) and quite similar to that of *B. subtilis* PfeT (3.25 ± 0.21 µmol/mg/h) (13). MtCtpD also showed significant activation at 1 mM Zn$^{2+}$ (Fig. 3B). Zn$^{2+}$ binding to P$_{1B4}$-ATPases as well as Zn$^{2+}$ transport has been reported (15, 30). The $K_{1/2}$ for Fe$^{2+}$ activation of the mycobacterial enzymes confirmed a tendency observed in *B. subtilis* PfeT: the larger activation by Fe$^{2+}$ is associated with a $K_{1/2}$ much larger than that of Co$^{2+}$ (Table 1). However, the observed $K_{1/2}$ values do not describe the selectivity to the enzymes. These parameters result from the $k_{on}/k_{off}$ of the metals binding the cytoplasmic facing transmembrane sites and the $k_{on}/k_{off}$ for the release/backward binding of the metal to the periplasmic facing sites (41). As shown below, equilibrium binding determinations of $K_D$ better report the relative selectivity for the activating metals.

The described Fe$^{2+}$-ATPase activities require the binding of the transported substrate to the TM-MBS. The stoichiometry of this interaction was verified by measuring Fe$^{2+}$ binding to MsCtpJ, MtCtpD, and MtCtpJ in non-turnover conditions lacking ATP (Table 1). The His$_6$-less enzymes were incubated with excess Fe$^{2+}$, unbound metal was removed by filtration, and bound metal was quantified by AAS. As expected, the proteins bind Fe$^{2+}$ in a 1:1 molar ratio. Discarding the possibility of nonspecific interactions, metal binding was largely abolished in the presence of 1.5 mM vanadate (not shown). This binding stoichiometry is similar to that previously observed for Zn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ binding to P$_{1B4}$-ATPases (15, 17). Notably, although the TM-MBSs of these enzymes appear to accommodate divalent cations, no significant binding of Co$^{3+}$ was observed (not shown).

Mycobacterial P$_{1B4}$-ATPase affinities for Fe$^{2+}$ and Co$^{2+}$ under equilibrium conditions were determined by titration of isolated enzymes in the presence of the fluorescence indicator mag-fura-2 (15, 42). In these experiments, mag-fura-2 forms 1:1 indicator-metal complexes of known $K_D$. The concentration of free indicator can be spectrophotometrically monitored, and the free metal and metal-protein complex levels can be calculated. The enzyme-metal $K_D$ and the apparent stoichiometry of the interactions were obtained by fitting mag-fura-2 $A_{565}$ versus free metal concentration curves (Table 1). MsCtpJ and MtCtpJ showed a similar $K_D$ for Fe$^{2+}$. These were also comparable with those previously reported for Co$^{2+}$ (included in Table 1 for comparison). Notably, MtCtpD has ∼3-fold higher affinity for Fe$^{2+}$ compared with Co$^{2+}$. Moreover, the affinity of MtCtpD is 20 times higher for Fe$^{2+}$ (lower $K_D$) and 5 times higher for Co$^{2+}$ when compared with those observed in the CtpJ enzymes. The relative preference of MtCtpD for Fe$^{2+}$ when compared with MtCtpJ further supports a dominant role of MtCtpD in Fe$^{2+}$ tolerance (Fig. 1).

**Distinct Co$^{2+}$ and Fe$^{2+}$ Coordination by MtCtpD—**Full appreciation of the different enzymatic activities and metal selectivity observed in P$_{1B4}$-ATPases requires understanding of the structural basis of these phenomena. P$_{1B4}$-ATPases share a number of invariant residues in the transmembrane region proposed to participate in metal coordination (19, 24–26). A six-coordinate Co$^{2+}$ species by the *Sulfitobacter* sp. P$_{1B4}$-ATPase has been postulated with participation of a Ser in the conserved SPC in the fourth TM and invariant His, Glu, and Thr in the
sixth TM (17). Surprisingly, this coordination did not include the invariant Cys located in the fourth transmembrane segment of all P1B-ATPases.

Considering the results shown above and the possible distinct Fe$^{2+}$/Co$^{2+}$ coordination, the binding environment of Fe$^{2+}$ and Co$^{2+}$ in MtCtpD was analyzed by XAS. The x-ray absorption near edge spectroscopy (XANES) portion of the XAS spectrum is element-specific and local bonding-sensitive; therefore it is useful for reporting the oxidation and coordination states of metals bound to the enzyme. The spectra of Fe$^{2+}$- and Co$^{2+}$-loaded protein were compared with Fe$^{3+}$ and Fe$^{3+}$ model systems (Fig. 4A). The first inflection point energy for protein-bound iron occurs at 7127.6 eV, consistent with a 50%/50% Fe$^{2+}$/Fe$^{3+}$ redox state mixture. Although all spectra were closely screened for photoreduction, iron oxidation during protein concentration postmetal loading may have led to this observation. Pre-edge features observed in the XANES of iron-MtCtpD are characteristic of 1$s$-3$d$ electronic transitions.

These pre-edge features are consistent with pseudosymmetric six-coordinate iron-ligand systems (43). Cobalt-MtCtpD XANES pre-edge features and the general edge structure are consistent with Co$^{2+}$ bound to protein systems in a six-coordinate ligand environment as observed previously (17, 44). The EXAFS region of an XAS spectrum provides high res-

**TABLE 1**

Summary of kinetic parameters, metal binding stoichiometry, and affinity of mycobacterial P$_{1B4}$-ATPases

<table>
<thead>
<tr>
<th></th>
<th><em>M. smegmatis</em> CtpJ</th>
<th><em>M. tuberculosis</em> CtpD</th>
<th><em>M. tuberculosis</em> CtpJ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATPase activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ (μmol/mg/h)</td>
<td>2.73 ± 0.12</td>
<td>4.25 ± 0.19</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>$K_{1/2}$ (μM)</td>
<td>350 ± 45</td>
<td>443 ± 54</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Metal stoichiometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_n$ (μM)</td>
<td>2.24 ± 0.43</td>
<td>0.12 ± 0.04</td>
<td>2.41 ± 0.27</td>
</tr>
<tr>
<td>$N$</td>
<td>1.44 ± 0.17</td>
<td>1.32 ± 0.11</td>
<td>1.35 ± 0.06</td>
</tr>
</tbody>
</table>

Values are the best fit parameters of activity versus Fe$^{2+}$ curves. Errors are asymptotic errors provided by the fitting software (Fig. 3D).

Values were previously reported (15, 16).

Values are the mean ± S.E. (n = 3).

Values obtained by competitive metal binding with mag-fura-2. Values are the mean ± S.D. (n = 3).

---

**FIGURE 3. Activation of mycobacterial P$_{1B4}$-ATPases by Fe$^{2+}$.** ATPase activity of purified MsCtpJ (A), MtCtpD (B), and MtCtpJ (C) in the presence of a 0.1 (green bars) or 1.0 mM (blue bars) concentration of the indicated metal ions was determined. D, Fe$^{2+}$ dependence of MsCtpJ (■; blue), MtCtpD (●; black), and MtCtpJ (●; green) ATPase activities. Data are the mean ± S.E. (error bars) of three independent experiments performed in duplicate.
The spectroscopy analysis points to a distinct coordination for Co\(^{2+}\) and Fe\(^{2+}\). However, the spectroscopy is not able to reveal alternative ligands. Conserved residues of MtCtpD possibly involved in metal coordination are Ser-316 and Cys-318 in TM4 and His-642, Glu-643, Gly-644, Ser-645, and Thr-646 in TM6. Seeking a more detailed understanding of the Fe\(^{2+}\) and Co\(^{2+}\) coordination at the TM-MBS, residues likely involved in the metal coordination were exchanged by site-directed mutagenesis, and the resulting proteins were functionally characterized. The single mutants E643D and T646S (including as conservative control modifications) and G644A and S645A did not alter the Co\(^{2+}\) or Fe\(^{2+}\)-ATPase activities of MtCtpD (Fig. 6). In agreement with previous reports, mutation of S316A, H642A, E643A, or T646A led to significant loss of Co\(^{2+}\) activity (6%) of WT activity) while preserving significant Fe\(^{2+}\) sensitivity (33%) at saturating metal concentrations. In contrast, the C318A mutation had diminished Fe\(^{2+}\) activation (18%) while retaining 39% of the Co\(^{2+}\)-ATPase activity. The differential effects of H642A and C318A mutations on the ATPase activity point toward a plausible mechanism. It appears that CtpD differentiates Co\(^{2+}\) and Fe\(^{2+}\) as substrates perhaps via alternative coordination despite binding these ions with quite similar affinities.

Considering that the ATPase activities might be affected by the removal of a ligating group or by the inability to undergo structural changes required for transport, the capability of the C318A and H642A mutant proteins to bind Co\(^{2+}\) and Fe\(^{2+}\) was tested. In comparison, the C318A mutant showed significantly lowered MtCtpD affinity for Fe\(^{2+}\), but it did not change the binding affinity for Co\(^{2+}\), suggesting an important role of this conserved Cys in Fe\(^{2+}\) binding (Fig. 7 and Table 3). The critical role of Cys-318 was further confirmed by the determination of the metal binding by AAS after incubation of the C318A mutant protein with metals at concentrations 10 times over the observed \(K_D\). The C318A mutant protein was able to bind 1.05 ± 0.14 Co\(^{2+}\) but only 0.32 ± 0.06 Fe\(^{2+}\). Finally, the H642A mutant had no detectable effect on Fe\(^{2+}\) or Co\(^{2+}\) binding affinities compared with WT (Table 3), raising the possibility that His-642 has a role other than the direct participation in the TM-MBS.

**Discussion**

The substrates and consequent functional roles of bacterial and eukaryotic P\(_{1\beta4}\)-ATPases have remained elusive as their capabilities to transport different transition metals have been reported (15–17, 28–30). This is relevant as some of these transporters are required for bacterial virulence (16, 18) and critical for metal homeostasis in chloroplasts (29, 46). An extra layer of complexity is present in bacterial systems containing homologous non-redundant P\(_{1\beta4}\)-ATPases (16). From a different perspective, defining P\(_{1\beta4}\)-ATPases substrates is significant to understanding the coordination of transition metals by...
transport proteins as well as likely additional selectivity mechanisms acting in vivo. Here we describe the roles of mycobacterial P14B-ATPases in Fe$^{2+}$/H$^{1001}$ homeostasis, the kinetics of transport, and the structural elements that in part determine the selectivity of these enzymes. These results indicate that mycobacterial P14B-ATPases are Fe$^{2+}$/H$^{1001}$/Co$^{2+}$/H$^{1001}$-ATPases; however, the various isoforms show differential participation in the homeostasis of these ions.

**Mycobacterial P$_{14B}$-ATPases Participates in Both Fe$^{2+}$ and Co$^{2+}$ Homeostasis**—We observed that mycobacterial CtpJ proteins contribute to the homeostasis of Fe$^{2+}$ and Co$^{2+}$ to different extents. *M. smegmatis* has a single P$_{14B}$-ATPase, MsCtpJ. Expression of the coding gene is induced by Co$^{2+}$/H$^{1001}$ and partially by the superoxide generator paraquat but not by H$_2$O$_2$ (15). Deletion of MsctpJ leads to lower tolerance to Co$^{2+}$, Fe$^{2+}$, and hemin as well as increments in intracellular Co$^{2+}$ and Fe$^{2+}$

<table>
<thead>
<tr>
<th>Nearest neighbor ligand environment$^a$</th>
<th>Long range ligand environment$^a$</th>
</tr>
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<tbody>
<tr>
<td>Metal</td>
<td>Atom$^b$</td>
</tr>
<tr>
<td>Iron</td>
<td>Oxygen/nitrogen</td>
</tr>
<tr>
<td></td>
<td>Oxygen/nitrogen</td>
</tr>
<tr>
<td>Cobalt</td>
<td>Oxygen/nitrogen</td>
</tr>
<tr>
<td></td>
<td>Oxygen/nitrogen</td>
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</tbody>
</table>

$^a$ Independent metal-ligand scattering environment.
$^b$ Scattering atoms.
$^c$ Average metal-ligand bond length.
$^d$ Average metal-ligand coordination number.
$^e$ Average Debye-Waller factor (Å$^2$).
$^f$ Number of degrees of freedom weighted mean square deviation between data and fit.
levels (Fig. 1) (15). The *M. tuberculosis* genome encodes two P$_{1B4}$-ATPases. MtCtpJ expression, like MsCtpJ, is induced by Co$^{2+}$ and to a lesser extent by redox stressors and Fe$^{2+}$ (16). The *MtActpJ* strain accumulates higher levels of both Co$^{2+}$ and Fe$^{2+}$ (Fig. 1) (16), again in a fashion similar to that of the *MsActpJ* strain. These characteristics appear similar to those observed for PfteT, the single P$_{1B4}$-ATPase present in *B. subtilis* (13). The comparable functions suggested by the observed phenotypes correlate with the analogous biochemistry of MsCtpJ, MtCtpJ, and *B. subtilis* PfteT. These three ATPases transport Fe$^{2+}$ and Co$^{2+}$ with surprisingly similar $V_{max}$ and $K_{1/2}$ for activation. Moreover, both CtpJs bind Fe$^{2+}$ and Co$^{2+}$ with micromolar affinities (equilibrium binding determinations have not been performed for PfteT). These affinities explain the observed capability of these enzymes to influence the cellular response to STN when extracellular iron is maintained at just 10 $\mu$M. More importantly, 2–3 $\mu$M Fe$^{2+}$ affinities appear consistent with reported free Fe$^{2+}$ levels in the 1–10 $\mu$M region (4). In fact, the iron-sensing transcriptional regulators Fur and IdeR have 9 $\mu$M $K_D$ for Fe$^{2+}$ (47, 48), indicating that these regulators are sensitive to the same concentration of Fe$^{2+}$ as the P$_{1B4}$-ATPases. Consequently, efflux CtpJ ATPases and influx transporter regulators are likely to coordinately respond to changes in metal levels not only under Fe$^{2+}$ stress conditions but also under normal conditions.

Homologous P$_{1B4}$-ATPases Present in Mycobacterial Genomes Have Distinct Roles—*M. tuberculosis*, as in other mycobacteria, has an additional P$_{1B4}$-ATPase, CtpD. Notably, MtCtpD, but not MtCtpJ, is required for bacterial virulence. What unique function does CtpD provide? The *MtActpD* strain is more sensitive to iron stress and accumulates higher levels of this metal than *MtActpJ*. The phenotypic differences between the *MtActpD* and *MtActpJ* strains may have a molecular basis either in the biochemistry of these enzymes or the iron pool that they transport. Notably, the phenotype of the *MtActpD:ActpJ* double mutant strain is similar to that of the *MtActpD* cells, suggesting that CtpD and CtpJ use the same iron pool as a substrate, and this can be controlled by MtCtpD alone. Alternatively, the molecular activities of MtCtpD and MtCtpJ appear distinct. MtCtpD has significantly higher Fe$^{2+}$-ATPase activity. Moreover, if the relative activation induced by Fe$^{2+}$/Co$^{2+}$ is considered, MtCtpD shows Fe$^{2+}$ activity 12 times larger than that generated by Co$^{2+}$. On the contrary, MtCtpJ shows higher activation by Co$^{2+}$ than MtCtpD and only a 1.5 Fe$^{2+}://$ATPase: Co$^{2+}$-ATPase ratio. Although these relative activities approximately correlate with the observed phenotypes, the higher affinity of MtCtpD for Fe$^{2+}$ ($K_D$ of 0.1 $\mu$M) appears to confer its dominant role in Fe$^{2+}$ homeostasis. This $K_D$ is 1 order of magnitude smaller than that reported for Fe$^{2+}$-sensing transcriptional regulators of influx systems (47, 48).

Distinct from CtpJ, CtpD is not induced by divalent metals but by redox stressors, such as the nitric oxide generator nitroprusside and the respiratory poison cyanide (16). In fact, the region upstream of *ctpD* contains the TTAG XXXXTCXXG operator sequence for the redox-sensing MtFurA regulator (49). Considering the release of Fe$^{2+}$ from iron-sulfur and mononuclear iron-containing proteins upon redox stress, it can be hypothesized that CtpD constitutes an early response to Fe$^{2+}$ dyshomeostasis that is independent of efflux (CtpJ), storage (bacterioferritin), and regulators (IdeR) that respond to higher free Fe$^{2+}$ levels.

The Coordination of Fe$^{2+}$ by P$_{1B4}$-ATPases Likely Requires the Invariant Cys in the Fourth TM—Metal selectivity is central to the physiological roles of P$_{1B}$-ATPases. In early studies, invariant Cys in the sixth TM (fourth TM in P$_{1B4}$-ATPases) were instrumental in defining P$_{1B}$-ATPases. Detection of other conserve residues in the transmembrane region led to the identification of P$_{1B}$-ATPases subgroups (19). The participation of these signature residues in the binding sites of P$_{1B1}$ Cu$^{2+}$-ATPases and P$_{1B2}$ Zn$^{2+}$-ATPases was later established (25, 26). Then it was relevant to establish the metal coordination in P$_{1B4}$-ATPases. Previous studies proposed that P$_{1B4}$-ATPases coordinate Co$^{2+}$ with a Ser in the conserved SPC in the fourth TM and invariant His, Glu, and Thr in the sixth TM of these proteins (17). Surprisingly, no participation of the archetypical Cys in the fourth TM in Co$^{2+}$ coordination was observed. However, a different coordination of Fe$^{2+}$ by MtCtpD might explain its distinct biochemistry, i.e. higher affinity for Fe$^{2+}$ and Co$^{2+}$ and higher activity in the presence of Fe$^{2+}$. We studied the coordination of Fe$^{2+}$ and Co$^{2+}$ while bound to MtCtpD TM-MBS by XAS and functionally analyzed variants carrying mutations in putative coordinating groups. XAS data indicate that both Fe$^{2+}$ and Co$^{2+}$ are coordinated by five to six oxygen/nitrogen ligands in a manner similar to that described previously for the Sulfitobacter sp. P$_{1B4}$-ATPase. That is, the spectroscopy does not
show the participation of sulfur atoms from the invariant Cys in the fourth TM as a metal ligand. Notably, mutagenesis studies showed an alternative portrait of MtCtpD TM-MBS. As shown in the case the Sulfitobacter sp. P1B4-ATPase, we observed that mutation of S316A, H642A, E643A, and T646A led to an almost complete inhibition of Co2\(^+\) activation, whereas replacement C318A retains significant (39%) Co2\(^+\)-ATPase activity. A different pattern is observed, however, for the effects of these mutations on the Fe2\(^+\)-ATPase. In this case, H642A retains some activity, whereas C318A causes a larger decrease in the activation by Fe2\(^+\). Although these differences are not dramatic, they suggest a putative differential involvement of these residues. Determination of the equilibrium binding affinities provided a more detailed view. Surprisingly, mutation H642A did not affect the metal binding to MtCtpD, suggesting that the reduced V_max of this mutant is associated with alterations in rate-limiting conformational steps rather than ion coordination. Keep in mind that metal release is the rate-limiting step in P-type ATPases (21, 50). More remarkably, replacement of C318A leads to a large reduction in the affinity for Fe2\(^+\) without affecting Co2\(^+\) binding. This datum in itself does not show a role of the conserved Cys in coordinating metals but suggests a direct effect, perhaps steric or through the second coordination sphere, in determining the affinity for Fe2\(^+\). In this case, the conservation of this Cys in the CPS signature sequence appears to be a logical consequence of the need to maintain a high binding affinity for Fe2\(^+\).

In summary, our observations suggest that mycobacterial P1B4-ATPases play a central role in Fe2\(^+\) homeostasis. CtpD in particular, likely regulated by FurA, constitutes part of the cellular response to redox-induced damage of iron centers.

**Author Contributions**—S. J. P. conducted most of the experiments, analyzed the results, and wrote the initial draft of the manuscript. B. E. L. performed X-ray spectroscopy analysis. J. E. L. and S. N. conducted growth and metal tolerance experiments using *M. tuberculosis* strains. C. M. S. oversaw in vivo experiments with *M. tuberculosis* and participated in manuscript revision. T. L. S. supervised x-ray spectroscopic analysis and participated in manuscript revision. J. M. A. conceived the idea for the project, directed the project, and wrote the paper with S. J. P.

**Acknowledgments**—We thank Andrew Baez (Worcester Polytechnic Institute) for assistance with early experiments. X-ray absorption spectroscopic studies were performed at the Stanford Synchrotron Radiation Lightsource (SSRL). SSRL is a national user facility operated by Stanford University on behalf of the United States Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program.

**References**


Fine-tuning of Substrate Affinity Leads to Alternative Roles of *Mycobacterium tuberculosis* Fe²⁺-ATPases

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doi: 10.1074/jbc.M116.718239 originally published online March 28, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.718239

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