THE ACTIVITY AND COFACTOR PREFERENCES OF N-ACETYL-1-D-MYO-INOSITYL-2-AMINO-2-DEOXY-\(\alpha\)-D-GLUCOPYRANOSIDE DEACETYLASE (MSHB) CHANGE DEPENDING ON ENVIRONMENTAL CONDITIONS

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Running title: MshB activity and cofactor preferences

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Actinomycetes, such as Mycobacterium species, are Gram-positive bacteria that utilize the small molecule mycothiol (MSH) as their primary reducing agent. Consequently, the enzymes involved in MSH biosynthesis are targets for drug development. The metal-dependent enzyme N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-\(\alpha\)-D-glucopyranoside deacetylase (MshB) catalyzes the hydrolysis of N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-\(\alpha\)-D-glucopyranoside (GlcNAc-Ins) to form 1-D-myo-inositol-2-amino-2-deoxy-\(\alpha\)-D-glucopyranoside (GlcN-Ins) and acetate, the fourth overall step in MSH biosynthesis. Inhibitors of metalloenzymes typically contain a group that binds to the active site metal ion, thus a comprehensive understanding of the native cofactor(s) of metalloenzymes is critical for the development of biologically effective inhibitors. Herein we examine the effect of metal ions on the overall activity of MshB, and probe the identity of the native cofactor. We find that the activity of MshB follows the trend Fe\(^{2+}\) > Co\(^{2+}\) > Zn\(^{2+}\) > Mn\(^{2+}\), Ni\(^{2+}\). Additionally, our results show that the identity of the cofactor bound to purified MshB is highly dependent on the purification conditions used (aerobic vs. anaerobic), as well as the metal ion content of the medium during protein expression. MshB prefers Fe\(^{2+}\) under anaerobic conditions regardless of the metal ion content of the medium, and switches between Fe\(^{2+}\) and Zn\(^{2+}\) under aerobic conditions as the metal content of the medium is altered. These results indicate that the cofactor bound to MshB under environmental conditions is dependent on environmental conditions, suggesting that MshB may be a cambialistic metallohydrolase that contains a dynamic cofactor. Consequently, biologically effective inhibitors will likely need to dually target Fe\(^{2+}\)-MshB and Zn\(^{2+}\)-MshB.

Actinomycetes, such as Mycobacterium species, do not have glutathione. Instead, these organisms use the small molecule mycothiol (MSH) as their primary reducing agent and in xenobiotic metabolism for the detoxification of drugs and other toxins (1-4). MSH is likely to be critical for the survival of mycobacteria inside activated macrophages, where the mycobacteria are subjected to oxidative bursts. Consequently, the enzymes involved in MSH biosynthesis and detoxification (Figure 1A), including the metalloenzymes MshB and mycothiol-conjugate amidase (MCA), are targets for the development of antibiotics for the treatment of diseases such as tuberculosis (5-10).

The enzyme MshB catalyzes the hydrolysis of GlcNAc-Ins to form GlcN-Ins and acetate, the fourth overall step in MSH biosynthesis (rate-limiting step (11)). MshB is an attractive drug target because it is a metalloenzyme; there are past successes in targeting metalloenzymes, including inhibitors of carbonic anhydrase, matrix metalloproteases, and angiotensin converting enzyme (12-15). Inhibitors of metalloenzymes typically contain a group that binds to the catalytic metal ion. Consequently, a comprehensive understanding of metalloenzyme cofactor preferences is necessary for the development of potent and specific metalloenzyme inhibitors.

MshB was previously identified as a Zn\(^{2+}\)-dependent enzyme based on the observations that the enzyme copurifies with Zn\(^{2+}\) (Figure 1B) and enzyme activity is reversibly inhibited by treatment with 1,10-phenanthroline (16-18). On the basis of the structure of the enzyme active site, MshB is thought to catalyze the hydrolysis of GlcNAc-Ins via one of two potential chemical
mechanisms using general acid-base catalysis (GABC) (19). One possible mechanism uses a single bifunctional GABC to facilitate the hydrolysis of GlcNAc-Ins, while the other uses a pair of GABC to carry out this reaction. However, Fe$^{2+}$ was not examined as a potential cofactor in these experiments. Furthermore, MshB was purified using Zn-IMAC under aerobic conditions, which is biased towards Zn incorporation into metalloenzymes (16). Purified MshB contains Ni (0.82 equiv.) when purified using Ni-IMAC (aerobic conditions) (16). There have been several examples over the last decade of Fe$^{2+}$-enzymes being misidentified as exclusive Zn$^{2+}$-enzymes, including peptide deformylase (PDF), LuxS, LpxC and possibly HDAC8 (20-27). In all these enzymes, the Fe$^{2+}$ cofactor is either exclusively preferred, or is preferred over Zn$^{2+}$ under certain environmental conditions. The incorporation of Zn$^{2+}$ into these enzymes that led to the initial characterizations as zinc-enzymes is attributed to purification of the enzymes under aerobic conditions, which leads to oxidation of Fe$^{2+}$, dissociation of Fe$^{3+}$ from the enzyme, and replacement with Zn$^{2+}$. The finding that the zinc ion observed in the crystal structure of MshB is a 5-coordinate metal ion (28) suggests that Fe$^{2+}$ could also possibly serve as cofactor for MshB since Fe$^{2+}$ prefers coordination numbers of 5-6 (19). Consequently, we have examined whether Fe$^{2+}$ can serve as an efficient cofactor for MshB.

Herein we demonstrate that the activity of MshB is ~3-fold higher with Fe$^{2+}$ as a cofactor compared to Zn$^{2+}$. The activity of Fe$^{2+}$-MshB is air-sensitive, while the activity of Zn$^{2+}$-MshB is stable under aerobic conditions. Additionally, we demonstrate that MshB preferentially binds Fe$^{2+}$ over Zn$^{2+}$ when purified under anaerobic conditions and when purified under aerobic conditions in the absence of added Zn$^{2+}$. Although MshB exhibits a higher affinity for Zn$^{2+}$ over Fe$^{2+}$, it is likely that higher [Fe$^{2+}$]$_{free}$ accounts for the cofactor preferences that are observed in these experiments. These results suggest that MshB likely uses Fe$^{2+}$ and Zn$^{2+}$ as biological cofactors under different environmental conditions. These results may have important biological implications in light of the dynamic changes in Zn and Fe concentrations that occur during the course of infection and suggest that biologically effective inhibitors will need to dually target Fe$^{2+}$-MshB and Zn$^{2+}$-MshB.

**MATERIALS AND METHODS**

*General Procedures*

All solutions were prepared using milliQ water. Primers were purchased from Integrated DNA Technologies. Genomic DNA was purchased from ATCC. DNA sequencing was performed at the Virginia Bioinformatics Institute DNA Sequencing Facility (Virginia Tech). Plasmids and PCR products were purified using the Wizard Plus SV Minipreps DNA Purification System and Wizard SV Gel and PCR Clean-up kits (Promega), respectively. All chemicals were purchased from ThermoFisher Scientific, Sigma-Aldrich and Gold Biotechnology. For all kinetic and thermodynamic experiments, solutions were prepared with reagents that did not contain extraneous metal ions and/or were treated with Chelex (Biorad), and solutions were stored in “metal-free” plasticware. For Fe$^{2+}$ experiments, a 400 mM FeCl$_2$ stock was prepared in 10 mM dithionite and diluted to 100 mM with 1X assay buffer prior to incubation with apo-MshB. Similarly, a 100 mM ZnSO$_4$ solution was prepared in 50 mM HEPES, pH 7.5 and diluted to 100 mM with 1X assay buffer prior to incubation with apo-MshB. To maintain anaerobic conditions, experiments were carried out either in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) and/or using assay buffers containing 10 mM tris (2-carboxyethyl) phosphine (TCEP) and completed in < 2 h to ensure that MshB was maximally active during the course of the assays. The concentrations of metal ions were measured by ion chromatography (IC) on an ICS-3000 (Dionex).

*Cloning*

The MshB genes from *M. smegmatis* and *M. tuberculosis* were cloned into expression vectors using the Flexi® technology (Promega). The expression plasmids used in these studies yield recombinant proteins containing an N-terminal affinity tag linked to MshB via a TEV protease site: pVP55A (His-tag)(29), pVP56K (His-MBP-tag)(30) and pFN18K (Halo-tag, Promega). The MshB genes were amplified from genomic DNA with *Pme* and *Sgf* restriction sites.
at the 5' and 3' ends, respectively. PCR products were digested with Flexi enzyme blend (Pmel and SgfI), and ligated into Flexi digested expression plasmids with T4 Ligase (New England Biolabs). For MtMshB, which contains an internal SgfI site, the PCR product was first ligated into a pZeroBlunt vector (Stratagene), the internal restriction site was removed by introducing a silent mutation using the Quik Change Lightening Site-Directed Mutagenesis Kit (Stratagene), and the MshB gene was liberated by Flexi digest prior to ligation into the Flexi digested pVP55A, pVP56K and pFN18K vectors. The plasmid sequences were verified by DNA sequencing.

**Protein Expression and Purification**

For large scale production of recombinant proteins, the pHis-MsMshB or pHisMBP-MtMshB plasmids were transformed into BL21(DE3) cells. Cells were grown in LB supplemented with Ampicillin (100 µg/mL) or Kanamyacin (50 µg/mL) at 37 °C with shaking (250 rpm) until an OD600 of ~0.6 was reached. Protein expression was induced with the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG), the temperature decreased to 25 °C and the cells were incubated with shaking overnight. After 4-14 hours, cells were harvested by centrifugation, resuspended in Buffer A (30 mM HEPES, 150 mM NaCl, 0.5 mM imidazole, pH 7.5), and stored at -80 °C.

Cells were lysed using an Emulsiflex-C3 high-pressure homogenizer (Avestin). The cell lysate was clarified by centrifugation (18,000 rpm, 4 °C) and loaded onto a pre-equilibrated (Buffer A) Co-IMAC or Ni-IMAC column (50 mL Chelating Sepharose, GE Healthcare charged with NiCl2 or CoCl2). MshB was purified at 4 °C. The column was washed with 150 mL Buffer A, and His-MshB or His-MBP-MshB was eluted using an imidazole step-gradient (200 mM ea: Buffer A + 10mM imidazole, Buffer A + 25 mM imidazole, Buffer A + 300 mM imidazole). Fractions containing His-MshB or His-MBP-MshB (via SDS-PAGE) were combined, concentrated (Amicon Ultra-15 centrifugal devices, Millipore) and dialyzed (Snakeskin tubing MWCO 10 K, Pierce) vs. 2 x 4 L Buffer A overnight in the presence of His-TEV protease (300 µg/mL) to remove the His- or His-MBP-tag. The resulting TEV cleaved protein was loaded onto a pre-equilibrated (Buffer A + 25 mM imidazole) Co-or Ni-IMAC column. The His-MBP and His-TEV remain bound to the Co-IMAC column, while MshB elutes in the flow through. Fractions containing MshB (via 12% SDS-PAGE) were combined, concentrated, and dialyzed vs. 2 x 4 L of 25 mM HEPES, 1.5 mM TCEP pH 7.5 (Slide-a-Lyzer MWCO 10K, Pierce). Protein concentration was determined using the Bradford Assay (Pierce or Sigma). Protein aliquots were flash frozen in liquid Nitrogen and stored at -80 °C. Protein identity was confirmed via mass spectroscopy (Keith Ray and Rich Helm, Virginia Tech) following digest with trypsin and peptide sequencing (MALDI-TOF-TOF). The resulting peptide sequences were analyzed using Matrix Science Mascot and confirmed the identity of MshB from *M. smegmatis* and *M. tuberculosis*, respectively, with peptide sequence coverage of 33% (MsMshB) and 44% (MtMshB). (sequences provided in supplemental Figure S1)

**Molecular Weight Determination**

The solution molecular weight of MshB was examined using size exclusion chromatography (supplemental Figure S2) (31). Purified enzyme was loaded onto a Superdex 200 10/300 GL (GE Healthcare) column pre-equilibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.5. The elution volumes were used to calculate the $K_a$ values ($K_a = (V_e-V_0)/(V_t-V_0)$, where $V_0$ is the void volume of the column, $V_e$ is the total volume of the column, and $V_t$ is the elution volume of the protein. A standard curve was prepared by plotting log Molecular Weight vs. $K_a$ using the following protein standards (GE Healthcare): aprotinin, 6.5 kDa; ribonuclease A, 13.7 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 44 kDa; and conalbumin, 75 kDa.

**Preparation of Apo-MshB and Metal-Reconstitution**

As purified, MshB contains bound metal ions and the metal ion content of purified MshB varies depending on the nature of the purification used (see supplemental Table S1). Consequently, for our studies we prepared apo-MshB and then selectively reconstituted the enzyme with the desired metal ions. For the preparation of apo-MshB, purified protein (≤ 100 µM) was incubated with 10 mM HEPES, 20 mM dipicolinic acid (DPA), 250 µM ethylenediamine tetraacetic acid
(EDTA), pH 7.5 on ice. After 1 hour, the protein solution was concentrated, washed (diluted with buffer and then concentrated) with 3 x 15 mL 25 mM HEPES, 1.5 mM TCEP pH 7.5 and run over a desalting column to remove residual DPA/EDTA. Metal ion concentrations were determined using an ICS-3000 (Dionex). Apo-MshB samples contained ≤ 10% metal/protein (supplemental Table S2).

Prior to activity measurements, apo-MshB (≤ 10 µM) was incubated with a stoichiometric concentration of the desired metal ion (CoCl2, FeCl2, FeCl3, MnCl2, NiCl2, ZnSO4) and incubated on ice 30 min. For experiments examining the optimal metal/protein ratio, apo-MshB was incubated with various concentrations of the desired metal ion (0 to 20 µM) for 30 min on ice prior to activity assay.

**MshB Deacetylase Activity**

MshB deacetylase activity was measured with the substrate N-Acetyl-glucosamine (GlcNAc, Sigma) using a fluorescamine (FSA)-based assay (32). In general, assay mixtures containing 50 mM HEPES, 50 mM NaCl pH 7.5 and 0-150 mM GlcNAc were pre-equilibrated at 30 °C, and the reactions were initiated by the addition of enzyme (1 µM). For pH-dependence experiments, the following buffers (all 50 mM containing 1 mM TCEP) were used: MES, pH 6-6.8; Mops, pH 6.5-7.5; HEPES, pH 7.3-8.8; Bicine, pH 8-9; Borate, pH 9-10; carbonate, pH 10-11. After incubation for various times, reactions aliquots (30 µL) were quenched by the addition of 20 % tricholoroacetic acid (TCA, 10 µL), and the cleared supernatant (25 µL) was transferred into a 96-well plate, diluted with 1 M borate pH 9 (75 µL) and reacted with FSA (30 µL in CH3CN, Invitrogen). After 10 minutes, the fluorescence was measured (Ex. 395 nm, Em. 485 nm) using a SpectraMax M5 platereader (Molecular Devices). Initial rates of product formation (< 10%) were determined from these data. Equation 1 was fit to the pH rate profile where \( k_1 \) represents \( V/K \) at the pH optimum, and \( K_{a1} \) and \( K_{a2} \) represent the dissociation constants describing the two ionizations.

\[
\frac{V}{K_{obs}} = \frac{k_1}{1 + [H^+] + \frac{K_{a2}}{K_{a1}}} [H^+]
\]

For determination of the steady-state parameters, deacetylase activity was measured at 6-8 different concentrations of GlcNAc (0 – 150 mM), and the parameters \( k_{cat} \), \( K_M \), and \( k_{cat}/K_M \) were obtained by fitting the Michaelis-Menten equation to the initial linear velocities using the curve-fitting program Kaleidagraph (Synergy Software), which also calculates the asymptotic standard errors.

**UV-Vis Spectrophotometry**

Apo-MsMshB (1 µM) was incubated with 1 µM FeCl2 or ZnSO4 in an anaerobic glovebox in 50 mM HEPES, 10 mM TCEP pH 7.5, for 30 minutes on ice to reconstitute the holo-enzyme. The enzyme solutions were transferred to sealed anaerobic cuvettes (Precision Cells) and the UV-Vis spectrum was recorded on an Agilent 8453 UV–visible spectrophotometer. The spectrum of the Zn2+-MshB sample was subtracted from the Fe2+-MshB to account for background absorbance attributed to the protein. The absorbance difference spectrum for Fe2+-MshB is shown in supplemental Figure S4.

**Halo-Tag Pulldown Experiments**

BL21(DE3) cells were transformed with pHalo-MshB, and grown in Chemically Defined Medium (CDM, 100 mL)(33) supplemented with kanamycin (50 µg/mL) at 37 °C with shaking (250 rpm) until an OD600 of ~0.6 was reached. Protein expression was induced by the addition of 1 mM IPTG along with the addition of either no added metals, 20 µM ZnSO4, 20 µM ferric ammonium citrate, or both metal supplements, and the cells were incubated overnight (12-14 h) with shaking (250 rpm) at 25 °C. Cells were harvested by centrifugation and washed with 5 mM CaCl2 (1 x 10 mL) and 10 mM Mops pH 7 (2 x 10 mL). Cell pellets were resuspended in 3 mL pull-down buffer (40 mM Mops, 150 mM NaCl, 10 mM TCEP, pH 7.5) and lysed by incubation with lysozyme (1 mg/mL) at room temperature for 15-30 minutes (Note: for anaerobic experiments, washed cell pellets were transferred into an
anaerobic chamber prior to resuspension in pull-down buffer). The cell lysate was cleared by centrifugation (15,000 rpm, 25-30 min.) and then incubated with 150 µL HaloLink™ Resin (Promega, pre-equilibrated in pull-down buffer) for 30 minutes. HaloLink™ Resin was washed with pull-down buffer (5 x 500 µL), resuspended in 250 µL pull-down buffer containing TEV protease (5 U/µL) and incubated at 37 °C for 45-60 minutes. The TEV protease expressed and purified in our laboratory is dialyzed against an EDTA-containing buffer, and therefore has a low metal content (<0.001 metal ion per protein). Cleaved MshB was separated from the resin by centrifugation (13,200 rpm, 2 min). The concentrations of metal ions were determined using the ICS-3000, and the concentration of MshB was determined using the Bradford Assay.

Metal Ion Affinity Experiments

The affinity of MshB for Zn(II) was determined using ultrafiltration as described for LpxC deacetylase (27). Briefly, apo-MshB (1 µM) was incubated in a metal ion and pH buffer containing 1 mM nitrioloacetic acid (NTA), 5 mM Mops pH 7, and 0 – 0.5 mM Zn_{total} (0 – 3.3 mM Zn_{free}) at 30 °C for ≥ 25 minutes. Free and bound metal ions were separated by centrifugation (1500 rcf x 5 min), and the concentration of metal ions (filtrate and retentate) was determined using IC. The $K_D$ value was determined by fitting a binding isotherm to these data.

The affinity of MshB for Fe(II) was determined by measuring the catalytic activity of MshB in the presence of varying concentrations of free Fe(II). Apo-MshB (1 µM) was incubated in a metal ion and pH buffer containing 1 mM NTA, 5 mM Mops pH 7 and 0 – 950 µM Fe(II)_{total} (0-2.6 µM Fe(II)_{free}) at 30 °C for ≥ 30 minutes in an anaerobic chamber. Activity assays were carried out as described above using 50 mM GlcNAc and 1 µM MshB. The $K_D$ value was determined by fitting a binding isotherm to these data.

Metal Ion Dissociation Rate Constants

The first order rate constant for metal ion dissociation from MshB was measured by determining the time-dependent loss of activity upon incubation of MshB with the chelator EDTA. Zn^{2+}-MshB or Fe^{2+}-MshB (50 µM) was diluted into assay buffer (50 mM HEPES, 50 mM NaCl, 1 mM TCEP, pH 7.5) containing 1 mM EDTA and incubated at 30 °C. After various times (0 – 200 min), an aliquot of enzyme was diluted 100-fold into assay buffer containing 20 mM GlcNAc, and the activity measured as described above. A single exponential decay was fit to the initial rates as a function of time to obtain the value $k_{off}$.

RESULTS

MshB is a mononuclear metalloenzyme

MshB was previously shown to undergo reversible inhibition by treatment with 1,10-phenanthroline, demonstrating that MshB is a metalloenzyme (16,28). However, determination of whether MshB is a mononuclear or binuclear metalloenzyme was not examined. Since Zn^{2+} is proposed to be the native cofactor for MshB, we examined the effect of varying the Zn^{2+}/MshB ratio on catalytic activity (Figure 2A). The results from these experiments demonstrate that MshB is maximally active with ~1 Zn^{2+} per MshB, indicating that MshB is a mononuclear metalloenzyme. This finding is consistent with the crystal structure of MshB that reveals a single bound zinc ion in the active site (Figure 1B) (28). Experiments on the titration of Fe^{2+} and Co^{2+} with MshB are also consistent with a mononuclear metalloenzyme (supplemental Figure S3).

The pH-dependence of the MshB catalyzed reaction is bell-shaped

The MshB catalyzed reaction (V/K conditions) exhibits a bell-shaped dependence on pH, indicating that there are two ionizations that are important for maximal catalytic activity (Figure 2B) with observed $pK_a$ values of 7.3 and 10.4 for Zn^{2+}-MshB. These results are consistent with MshB proceeding through either a single bifunctional GABC or a GABC pair mechanism, as observed for other metal-dependent deacetylases (19).

Fe^{2+}-MshB exhibits the highest activity

In previous experiments, the steady-state parameters for Zn^{2+}-MshB were determined using the substrates GlcNAc-Ins, MSmB, and CySmB-GlcN-Ins (16). Although it was stated that the activity of apo-MshB could be restored with Zn^{2+}, Ni^{2+}, Mn^{2+}, or Co^{2+}, no specific values for the rate
enhancements observed were reported and the steady-state parameters for MshB activity were only provided for Zn\textsuperscript{2+}-MshB. Since information about the chemical mechanism can be gained from the relative changes in activity upon reconstitution with different metal ions and Fe\textsuperscript{2+} had not been previously examined as a potential cofactor, we determined the steady-state parameters for MshB substituted with different metal ions (Table 1, Figure 2C). We chose to use the commercially available substrate GlcNAc in these experiments. GlcNAc has a weakened affinity for MshB compared to GlcNAc-Ins, however, the group that undergoes hydrolysis in these two substrates is the same (16,32).

The relative ability of various metal ions to activate MshB was measured. In these experiments, apo-MshB was reconstituted (stoichiometric) with the metal ion of interest, and the initial rates of product formation were measured at different substrate concentrations. Interestingly, we observe that MshB exhibits the highest activity with Fe\textsuperscript{2+} (Figure 2C), with the overall trend Fe\textsuperscript{2+} > Co\textsuperscript{2+} > Zn\textsuperscript{2+} > Mn\textsuperscript{2+} > Ni\textsuperscript{2+}. A closer examination of the steady-state parameters (Table 1) reveals that changes to the identity of the catalytic cofactor have only a minor effect on $K_M$ (< 2-fold), with much larger effects on $k_{cat}$ (8-fold) and $k_{cat}/K_M$ (~10-fold).

**Characterization of Fe\textsuperscript{2+}-MshB**

To confirm that the activation of MshB was specific for Fe\textsuperscript{2+}, and not Fe\textsuperscript{3+}, we measured the ability of Fe\textsuperscript{3+} to activate apo-MshB. The results in Figure 2C indicate that the activity of Fe\textsuperscript{3+}-MshB is comparable to apo-MshB (at stoichiometric metal:enzyme), suggesting that Fe\textsuperscript{3+} is not an efficient catalyst. Additionally, we examined the effect of oxygen on the activity of Fe\textsuperscript{3+}-MshB. Results from these experiments (Figure 2D) show that there is a time-dependent loss of activity for Fe\textsuperscript{3+}-MshB under aerobic conditions, while the activity of Fe\textsuperscript{2+}-MshB under anaerobic conditions is stable over a similar period of time. In contrast, the activity of Zn\textsuperscript{2+}-MshB is stable under aerobic conditions. We have also examined the absorbance spectrum of Fe\textsuperscript{3+}-MshB. The Fe\textsuperscript{3+}-MshB difference spectrum has a broad peak at 362 nm, consistent with an Fe-containing enzyme (supplemental Figure S4).

**The cofactor bound to MshB is dependent on environmental conditions**

Native cofactor identification is in large part defined by the metal ion that copurifies with the enzyme (19). Therefore, we developed a method that would allow us to rapidly purify MshB under various conditions using the Halo-tag® technology. The Halo-tag does not bind metal ions, and its rapid nature should prevent re-equilibration or switching of bound cofactors during the purification process.

Since previous experiments examining the identity of the cofactor bound to MshB relied on Ni- or Zn-IMAC purification,(16) which artificially introduce metal ions, we initially expressed MshB in LB medium and examined the cofactor bound to purified MshB under aerobic conditions using the Halo-tag approach (Figure 3, Supplemental Data Table S3). Under these conditions, MshB is isolated with Zn\textsuperscript{2+} as the predominant bound cofactor, consistent with what is observed following Zn-IMAC purification (16).

We also probed what happened to the identity of the cofactor bound to MshB when the protein was expressed under more stringent (metal ion) conditions. In these experiments, MshB was expressed in CDM ± Zn and/or Fe supplementation to examine the effect metal ion availability in the surrounding environment has on the identity of the bound cofactor, and MshB was purified under anaerobic and aerobic conditions to probe the effect of oxygen on the bound cofactor. Results from these experiments (Figure 3B) clearly indicate a strong preference for the Fe\textsuperscript{2+} cofactor under anaerobic conditions, regardless of the metal ion content of the medium. Additionally, these results suggest that the preferred cofactor changes between Zn\textsuperscript{2+} and Fe\textsuperscript{2+} depending on the metal ion content of the medium when MshB is purified under aerobic conditions. Since we used IC to measure the metal ions bound to MshB, we know that the Fe bound to MshB is Fe\textsuperscript{2+} and not Fe\textsuperscript{3+}, as these ions elute at different retention times (supplemental Figure S5).

**Zn\textsuperscript{2+} has a higher affinity than Fe\textsuperscript{2+} for MshB**

To determine if the preference we observed in the pull-down experiments could be explained by a higher affinity of Fe for MshB, we measured the affinity of Zn\textsuperscript{2+} and Fe\textsuperscript{2+} for MshB. The results from these experiments (Table 2,
Figure 4) show that Zn$^{2+}$ has a ~5300-fold higher affinity for MshB compared to Fe$^{2+}$ with $K_D$ values of 0.02 and 106 nM for Zn$^{2+}$ and Fe$^{2+}$, respectively.

To gain insights into whether the observed differences in metal ion affinity for MshB were due to differences in metal ion association and/or dissociation, we measured the dissociation constants for Zn$^{2+}$ and Fe$^{2+}$ from MshB (Table 2). The results from these experiments indicate that the dissociation of Zn$^{2+}$ and Fe$^{2+}$ from MshB are comparable (~0.03 min$^{-1}$). Consequently, the differences in the $K_D$ values are due to the faster association rate constant for Zn$^{2+}$ compared to Fe$^{2+}$, which are calculated ($K_D = k_{cat}/k_{on}$) to be 2.8 x 10$^7$ and 5.0 x 10$^3$ M$^{-1}$s$^{-1}$, respectively.

**DISCUSSION**

*MshB most active with Fe$^{2+}$ cofactor*

The data presented here indicate that MshB functions as a mononuclear metallohydrolase (Figure 2A). The activity of MshB follows the trend Fe$^{2+}$ > Co$^{2+}$ > Zn$^{2+}$ > Mn$^{2+}$, Ni$^{2+}$, which is inversely related to the Lewis acidity of the metals (with exception of Mn$^{2+}$). The finding that the highest activity is observed with Fe$^{2+}$-MshB is the first evidence to suggest that MshB may not be an exclusive Zn$^{2+}$-dependent enzyme as previously reported and raises the possibility that Fe$^{2+}$ may function as a biologically relevant cofactor thereby making MshB a cambialistic enzyme. Consistent with these data, we observe that MshB copurifies with Fe$^{2+}$ and Zn$^{2+}$ when expressed in CDM (lacking metal ion supplementation).

We have measured the steady-state parameters for MshB reconstituted with various metal ions. The results from these experiments indicate that there is no significant effect on $K_M$, which may suggest that substrate binding is not affected by changes in the identity of the metal ion. Significantly larger effects are observed on $k_{cat}$ and $k_{cat}/K_M$, suggesting that the rate of the chemical step may be altered by changes to the catalytic metal ion as expected for a metallohydrodrolase.

**Fe$^{2+}$-MshB may be subject to redox regulation**

The activity observed upon activation with Fe is due to Fe$^{2+}$, not Fe$^{3+}$, for the following reasons: 1) Fe$^{3+}$ cannot restore activity to apo-MshB (Figure 2C), 2) a time-dependent decrease in activity is observed for Fe$^{2+}$-MshB under aerobic conditions (Figure 2D), consistent with oxidation of Fe$^{2+}$ to Fe$^{3+}$, and 3) MshB copurifies with Fe$^{2+}$, not Fe$^{3+}$ (Figure 3B). These results suggest that the biological activity of Fe$^{2+}$-MshB may be regulated by redox changes in the surrounding environment. This possibility may have biological implications for pathogenic mycobacteria that reside inside macrophages where they are subject to oxidative bursts upon (macrophage) activation.

*Cofactor preferences are determined by metal ion availability*

We have examined the cofactor preferences of MshB for the first time where the protein is expressed under conditions of varying metal ion availability and the protein purified under anaerobic conditions using a new purification protocol that relies on the Halo-tag rather than IMAC purification. It is observed that MshB prefers Fe$^{2+}$ when isolated under anaerobic conditions, regardless of the metal ion content of the medium used to grow the cells (Figure 3B). Additionally, it was found that the cofactor bound to MshB under aerobic conditions switches between Zn$^{2+}$ and Fe$^{2+}$ depending on the metal ion availability of the medium used during the protein expression. Similar results have been observed with the metal-dependent deacetylase LpxC (supplemental Table S4) (27). In light of the observed activity data for MshB and LpxC, these results imply that at least a subset of metallohydrolases may be cambialistic enzymes that utilize multiple cofactors in vivo, and can use metal (cofactor) switching as a mechanism for regulation of enzyme activity in response to changing environmental conditions.

The affinity of MshB for Zn$^{2+}$ is ~5,300-fold greater than Fe$^{2+}$ (Table 2), indicating that the observed metal ion preferences are not dictated solely by metal ion affinity. Instead, the observed preference for Fe$^{2+}$ under anaerobic conditions appears to be dictated by the greater availability of Fe$^{2+}$ (estimated 10-400 pm Zn$^{2+}$, 0.2-6 µM Fe$^{2+}$) (27). Interestingly, the dissociation rate constants for Fe(II) and Zn(II) are comparable (0.03 min$^{-1}$), suggesting that the observed differences in affinity are primarily attributed to
metal ion association with MshB. It should be pointed out that the dissociation of Zn(II) from MshB does not go to an endpoint of zero. Similar findings have been reported for carboxypeptidase A (34,35), carbonic anhydrase (36) and to some extent LpxC (27), and may suggest that a fraction of the active site Zn(II) dissociates more rapidly. While the association rate constant for Zn(II) with MshB approaches that of a diffusion controlled process (2.8 x 10^7 M^{-1}s^{-1}), the association rate constant for Fe(II) is much slower than a diffusion controlled process (5 x 10^3 M^{-1}s^{-1}) and is more consistent with a two-step binding mechanism. Two-step binding mechanisms have been used to describe Zn(II) binding to carbonic anhydrase (37) and Fe(II) binding to LpxC (27).

**Significance of a Fe/Zn cambialistic MshB**

The results suggesting that MshB may be a cambialistic enzyme that can utilize either Fe^{2+} or Zn^{2+} as a cofactor are also interesting in light of the changes in Zn and Fe that occur in the mycobacterial environment (vacuoles of infected macrophages) during the course of infection. Metal imaging experiments were used to visualize changes in metal ion concentrations that occur in macrophages following both infection with mycobacteria and after activation of the infected macrophages (38). Results from these experiments indicate that the vacuoles (marker for the location of mycobacteria) of macrophages infected with *M. tuberculosis* contain a much higher concentration of Fe (3 mM) compared to Zn (450 µM). Furthermore, the results from these experiments indicate that Zn and Fe are the only divalent metal ions that undergo significant changes in concentration during the course of infection. Activation of macrophages infected with *M. avium* with TNF-α leads to a significant increase in Zn (0.12 mM to 1.8 mM) and decrease in Fe (1.2 mM to 0.27 mM) in the vacuoles. These dynamic changes to the Fe/Zn concentrations in the mycobacterial environment during the course of infection may have a large impact on metalloenzymes whose activity is altered by changes in Zn and Fe concentrations, such as MshB. Furthermore, the ability of MshB to function as a Fe/Zn cambialistic enzyme would enable MshB to adapt to changing environmental conditions, such as those encountered in the macrophages, and allow the organism to continue producing MSH throughout the course of infection when large changes in Fe/Zn concentrations occur. This could be one key factor that aids mycobacterial survival inside the macrophages. Additionally, these results suggest that biologically effective inhibitors will need to dually target Fe^{2+}-MshB and Zn^{2+}-MshB.

**SUPPLEMENTAL DATA**

Mass Spectroscopy peptide sequences, plots (size exclusion chromatography, Co^{2+} and Fe^{2+} stoichiometry, absorbance spectrum of Fe^{2+}-MshB, and sample IC trace) and tables of metal content of purified MshB are provided as supplemental data.

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Abbreviations: MSH, mycothiol; MshB, N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase; MCA, mycothiol-conjugate amidase; GlcNAc-Ins, N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside; GlcN-Ins, 1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside; GABC, general acid-base catalysis; IMAC, immobilized metal ion affinity chromatography; PDF, peptide deformylase; LuxS, S-ribosylhomocysteinase; LpxC, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylgalcosamine deacetylase; HDAC8, histone deacetylase 8; TCEP, triscarboxyethylphosphine; IC, ion chromatography; TEV, Tobacco Etch Virus; MBP, maltose binding protein; IPTG, isopropyl-β-D-thiogalactoside; DPA, dipicolinic acid; EDTA, ethylenediamine tetraacetic acid; CDM, chemically defined medium; PD, pull-down; GlcNAc, N-Acetyl-glucosamine; FSA, fluorescamine; TCA, trichloroacetic acid; MSmB, monobromobimane S-conjugate of mycothiol; CySmB-GlcN-Ins, bimane S-conjugate of Cys-GlcN-Ins.

FIGURE LEGENDS

Figure 1: (A) Reaction catalyzed by MshB (B) Active site of MshB (PDB 1Q74) containing a catalytic zinc ion.

Figure 2: Catalytic activity of MshB. (A) Activation of apo-MshB with (○) Zn^{2+}. Deacetylase activity was measured as a function of Zn/MshB stoichiometry. Apo-MshB was incubated with varying equivalents of Zn^{2+} (0-2). After 30 minutes, the enzyme was diluted into assay buffer containing substrate GlcNAc (50 mM) and the resulting deacetylase activity was measured at 30 °C as described in “Materials and Methods”. (B) pH dependence of the Zn^{2+}-MshB-catalyzed reaction. The V/K values were measured at 30 °C using subsaturating concentrations of GlcNAc (5 mM) as described in “Materials and Methods”. The pKₐ values of 7.3 and 10.4 were determined by fitting Eq. 1 to these data. (C) Steady-state turnover catalyzed by metal substituted MshB: (▽) apo-MshB, (■) Co^{2+}, (○) Zn^{2+}, (●) Fe^{2+}, (△) Fe^{3+}. Apo-MshB was incubated with stoichiometric amounts of metal ions. After 30 minutes, the enzyme was diluted into assay buffer containing substrate and the initial rates for the deacetylation of (GlcNAc, 0-150 mM) were measured at 30 °C as described in “Materials and Methods”. The steady-state parameters k_{cat}, K_M, and k_{cat}/K_M (Table 1) were obtained by fitting the Michaelis-Menten equation to the initial rates. (D) Fe^{2+}-MshB is activity is air sensitive. Apo-MshB was reconstituted with Fe^{2+} or Zn^{2+}, and the resulting deacetylase activity was measured at 30 °C at t = 0 (anaerobic, black bars). The enzyme was then either exposed to aerobic conditions for 3 hours (gray bars) or kept under anaerobic conditions for 3 hours (white bars) and the resulting deacetylase activity measured using the substrate GlcNAc (50 mM) as described in “Materials and Methods”.

Figure 3: Metal ion content of recombinant MshB isolated from E. coli using Halo-tag pull-down experiments. pHalo-MshB E. coli (BL21(DE3) cells) were grown in LB or CDM and induced with 1 mM IPTG with or without iron or zinc supplementation (20 μM). MshB was purified using Halo-tag pulldown experiments under aerobic and anaerobic conditions as described in “Materials and Methods”. (A) Purification of MshB using the Halo-tag. An aliquot of protein at each step of the purification was
analyzed on a 12% SDS-PAGE. MW Marker (Lane 1), cleared cell lysates containing Halo-tag fusion proteins (Lane 2 = MsMshB, Lane 6 = MtMshB), positive control (Lane 3 = MshB), *PD supernatant after TEV cleavage (Lane 4 = MsMshB, Lane 7 = MtMshB), and TEV only control (Lane 5). (B) The Fe/Zn ratio of metal ions bound to MshB following aerobic (black bars) and anaerobic (gray bars) purification was determined by IC as described in “Materials and Methods”.

**Figure 4:** Properties of metal ion binding to MshB (A) The metal ion affinity of MshB for (◯) Zn$^{2+}$ and (●) Fe$^{2+}$. Apo-MshB was equilibrated with buffered metal ion solutions (5 mM Mops, 1 mM NTA, pH 7 as described in “Materials and Methods”). The fraction bound Zn was determined using ultrafiltration and IC analysis, and the fraction bound Fe was determined by enhancement of MshB deacetylase activity using GlcNAc (50 mM) substrate (see “Materials and Methods”). A binding isotherm was fit to the resulting data to obtain $K_{D_{\text{Zn(II)}}}$ and $K_{D_{\text{Fe(II)}}}$ values (Table 2). (B) Metal ion dissociation from MshB. Holo-MshB reconstituted with (◯) Zn$^{2+}$ or (●) Fe$^{2+}$ was incubated in assay buffer (50 mM HEPES, 50 mM NaCl, 1 mM TCEP, pH 7.5) containing 1 mM EDTA, or 5 mM EDTA (◆, Zn$^{2+}$). At various times, the enzyme was diluted into assay buffer containing GlcNAc (50 mM) and the deacetylase activity measured at 30 °C as described in “Materials and Methods”. The $k_{off}$ values (Table 2) were obtained by fitting the a single exponential decay equation to these data.
### TABLES

**Table 1.** Catalytic activity of metal substituted MshB

| MshB<sup>a,b</sup> | $K_M$ (mM) | $k_{cat}$ (s<sup>-1</sup>) | $k_{cat}/K_M$ (M<sup>-1</sup> s<sup>-1</sup>) |
|-------------------|-----------|-----------------|------------------|
| Apo               | 36 ± 7    | 0.13 ± 0.01     | 3.5 ± 0.3        |
| Co<sup>2+</sup>   | 34 ± 5    | 1.1 ± 0.08      | 32 ± 2           |
| Fe<sup>2+</sup>   | 41 ± 5    | 2.1 ± 0.15      | 52 ± 3           |
| Fe<sup>3+</sup>   | 56 ± 13   | 0.11 ± 0.01     | 1.8 ± 0.3        |
| Mn<sup>2+</sup>   | 54 ± 13   | 0.38 ± 0.04     | 7.0 ± 1.0        |
| Ni<sup>2+</sup>   | 53 ± 14   | 0.27 ± 0.03     | 5.3 ± 0.8        |
| Zn<sup>2+ c,d</sup> | 38 ± 4   | 0.77 ± 0.04     | 20 ± 1           |

<sup>a</sup> apo-MshB was incubated with stoichiometric metal for 45 min prior to activity measurement; <sup>b</sup> Substrate used GlcNAc; <sup>c</sup> Data adapted from (32) <sup>d</sup> For comparison: (Substrate = GlcNAc-Ins) $K_M = 340 ± 80$ µM, $k_{cat} = 0.49 ± 0.04$ s<sup>-1</sup>, $k_{cat}/K_M = 1440 ± 360$ M<sup>-1</sup>s<sup>-1</sup> as reported in ref (16).
Table 2. Metal binding properties of MshB

| MshB  | $K_D$ (nM) $^a$ | $k_{off}$ (min$^{-1}$) | $k_{on}$ (M$^{-1}$s$^{-1}$) $^b$ |
|-------|----------------|-------------------------|-------------------------------|
| Fe$^{2+}$ | 106 ± 15 | 0.032 ± 0.006 | 5.0 x 10$^3$ |
| Zn$^{2+}$ | 0.02 ± 0.004 | 0.033 ± 0.001 | 2.8 x 10$^7$ |

$^a$ The affinity of MshB for Zn$^{2+}$ and Fe$^{2+}$ was determined using ultrafiltration or activity measurements in metal ion buffered solutions at 30 °C as described in “Materials and Methods”. $^b$ The association rate constants ($k_{on}$ values) for Zn$^{2+}$ and Fe$^{2+}$ were calculated from the $K_D$ and $k_{off}$ values.
FIGURES

Figure 1:

A

\[
\text{GlcNAc-Ins} \xrightarrow{\text{MshB}} \text{GlcN-Ins} \xrightarrow{\text{H}_2\text{O}, \text{AcO}^-} \text{Mycothiol}
\]

B

D15

H2O

H2O

H144

Zn

H13

H147

D146
Figure 2:

A

B

Relative activity

[Zn$^{2+}$] / [MshB]

V/K (mM$^3$ min$^{-1}$)

pH

0.01 0.1 1 10

6 7 8 9 10 11 12
Figure 3:
Figure 4:

A

B