EXAMINATION OF THE MECHANISM OF N-ACETYL-1-D-MYO-INOSITYL-2-AMINO-2-DEOXY-\(\alpha\)-D-GLUCOPYRANOSIDE DEACETYLASE (MshB) REVEALS AN UNEXPECTED ROLE FOR A DYNAMIC TYROSINE

Xinyi Huang and Marcy Hernick*†
Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061
Running title: Mechanism of MshB Deacetylase
*To whom correspondence should be addressed. Phone: (540) 231-2842; Fax: 540-231-9070; email: hernickm@vt.edu

Background: MshB is a metal-dependent deacetylase involved in mycothiol biosynthesis.
Result: The reaction proceeds via a general-acid-base pair mechanism and uses a dynamic Tyr that modulates substrate binding, chemistry, and product release.
Conclusion: The catalytic mechanism differs from a prototypical metalloprotease mechanism.
Significance: Key side chains identified in these studies can be targeted for inhibitor development.

SUMMARY
Actinomycetes are a group of Gram-positive bacteria that includes pathogenic Mycobacterial species such as M. tuberculosis. These organisms do not have glutathione, and instead utilize the small molecule mycothiol (MSH) as their primary reducing agent and for the detoxification of xenobiotics. Due to these important functions, enzymes involved in MSH biosynthesis and MSH-dependent detoxification are targets for drug development. The metal-dependent deacetylase N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-\(\alpha\)-D glucopyranoside deacetylase (MshB) catalyzes the hydrolysis of N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-\(\alpha\)-D glucopyranoside to form 1-D-myo-inosityl-2-amino-2-deoxy-\(\alpha\)-D glucopyranoside and acetate in MSH biosynthesis. Herein we examine the chemical mechanism of MshB. We demonstrate that the side chains of Asp15, Tyr142, His144, and Asp146 are important for catalytic activity. We show that NaF is an uncompetitive inhibitor of MshB, consistent with a metal-water/hydroxide functioning as the reactive nucleophile in the catalytic mechanism. We have previously shown that MshB activity has a bell-shaped dependence on pH with p\(K_a\) values of \(\sim 7.3\) and \(10.5\) (Huang, X., Kocabas, E. and Hernick, M. (2011) Journal of Biological Chemistry, 286, 20275-20282).

Mutagenesis experiments indicate that the observed p\(K_a\)’s reflect ionization of Asp15 and Tyr142, respectively. Together, findings from our studies suggest that MshB functions through a general acid-base pair mechanism with the side chain of Asp15 functioning as the general base catalyst and His144 serving as the general acid catalyst, while the side chain of Tyr142 likely assists in polarizing substrate/stabilizing the oxyanion intermediate. Additionally, our results indicate that Tyr142 is a dynamic side chain that plays key roles in catalysis - modulating substrate binding, chemistry, and product release.

INTRODUCTION
Actinomycetes, such as Mycobacterium species, are Gram-positive bacteria that contain a high GC-content and a thick, hydrophobic cell wall. Pathogenic Mycobacterial species are responsible for a number of infectious diseases, most notably tuberculosis and leprosy. In contrast to eukaryotes and other bacteria, these organisms do not have glutathione. Instead, they use the small molecule mycothiol (MSH)\(^1\) as their primary reducing agent and in xenobiotic metabolism for the detoxification of drugs and other toxins (1-4). MSH is likely critical for survival of mycobacteria inside the oxidative environment of activated macrophages where they reside. Consequently, enzymes in the MSH biosynthetic and MSH-dependent detoxification pathways are targets for the development of antibiotics for the treatment of diseases such as tuberculosis (5-10).

The metalloenzyme 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 (Figure 1a). MshB is an attractive drug target because it
MshB catalyzes the rate-limiting step in MSH biosynthesis (11), it is a metalloenzyme (12-14), and the three-dimensional structure is known (15,16). There are past successes in targeting metalloenzymes, including inhibitors of carbonic anhydrase, matrix metalloproteases, and angiotensin converting enzyme (17-20). Since inhibitors of metalloenzymes typically contain a group that binds to the catalytic metal ion, we previously examined the cofactor preferences of MshB and found that MshB is a cambialistic metalloenzyme whose in vitro activity follows the trend: Fe²⁺ > Co²⁺ > Zn²⁺ > Mn²⁺ > Ni²⁺ (14). Additionally, we found the cofactor bound to MshB is dependent on environmental conditions (14). MshB prefers Fe²⁺ under anaerobic conditions regardless of the metal ion content of the medium, and switches between Fe²⁺ and Zn²⁺ under aerobic conditions as the metal content of the medium is altered. MshB has a bell-shaped dependence on pH (sub-saturating concentrations of substrate, $V/K$) indicating that there are two ionizations that are important for maximal catalytic activity (14), consistent with a reaction that proceeds through either a single bifunctional general acid-base catalyst (GABC) or GABC pair mechanism (21).

Herein we probe the chemical mechanism of MshB. We demonstrate that residues D15, H144, D146, and Y142 are important for maximal catalytic activity. Our results suggest that D15 functions as a general base catalyst (GBC), while H144 functions as a general acid catalyst (GAC) and the catalytic metal-water/hydroxide serves as the reactive nucleophile in the reaction. Furthermore, our results indicate that Y142 is important for catalytic activity. We propose that Y142 is a dynamic side chain that modulates substrate binding, chemistry (via polarization of carbonyl group/stabilization of oxyanion), and product release. These insights into the chemical mechanism indicate that MshB does not follow the prototypical metalloprotease-like mechanism, but instead uses a GABC pair and a dynamics to catalyze the hydrolysis of substrate.

**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). All chemicals were purchased from ThermoFisher Scientific, Sigma-Aldrich and Gold Biotechnology. For kinetic 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. To maintain anaerobic conditions (Fe²⁺ assays), experiments were carried out in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Molecular graphics images were produced using the UCSF Chimera package (22).

**Protein Expression and Purification**

The previously reported plasmid encoding the MshB gene from *M. smegmatis* containing a N-terminal His-MBP tag was used as the template for preparation of mutant plasmids (14). All mutant plasmids were prepared using the Quick Change Lightning Site-Directed Mutagenesis Kit (Stratagene). Plasmid sequences were verified by DNA sequencing. All MshB variants were expressed and purified according to published procedures (14,23).

Briefly, cells were lysed using an Emulsiflex-C3 high-pressure homogenizer (Avestin) and MshB variants were purified at 4 °C. Cell lysate was clarified by centrifugation (18,000 rpm, 4 °C) and loaded onto a pre-equilibrated (Buffer A - 30 mM HEPES, 150 mM NaCl, 1mM TECP, 0.5 mM imidazole, pH 7.5) Co-IMAC column (50 mL Chelating Sepharose, GE Healthcare charged with CoCl₂). The column was washed with 150 mL Buffer A, and His-MBP-MshB was eluted using an imidazole step-gradient (200 mL ea: Buffer A + 30 mM imidazole, Buffer A + 25 mM imidazole, Buffer A + 25 mM imidazole, Buffer A + 300 mM imidazole). Fractions containing 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-MBP-tag. The resulting TEV-cleaved protein was loaded onto a pre-equilibrated (Buffer A + 25 mM imidazole) Co-IMAC column. 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 (Sigma). Protein aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

For the preparation of apo-MshB (14), 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. 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 for 30 min.

**MshB Deacetylase Activity**

MshB deacetylase activity was measured with the substrate N-acetyl-glucosamine (GlcNAc, Sigma) using a fluorescamine (FSA)-based assay (23). While the GlcNAc substrate has a decreased affinity for MshB compared to the natural substrate GlcNAc-Ins (Kₘ 38 mM vs. 340 μM), the GlcNAc moiety that undergoes the chemical transformation is conserved (12,23). Since our primary interest is on examining the chemical step of the reaction, the commercially available GlcNAc substrate was used in these studies. In general, assay mixtures containing 50 mM HEPES, 50 mM NaCl, 1 mM TCEP, pH 7.5 and 0-150 mM GlcNAc were pre-equilibrated at 30 °C, and reactions were initiated by the addition of enzyme (1 μM). For pH-dependence experiments, the following buffers were used (all 50 mM containing 1 mM TCEP, 50mM NaCl): MES, pH 6-6.8; Mops, pH 6.5-7.5; HEPES, pH 7.3-8.8; Bistris propane, 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% trichloroacetic 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 k₁ represents V/K at the pH optimum, and Kₐ₁ and Kₐ₂ represent dissociation constants describing the two ionizations. Equations 2 and 3 were fit to the pH rate profiles for the D15A and Y142A/F mutants, respectively, in which only a single ionization is observed. For experiments under V/K conditions, 5-50 mM GlcNAc was used as the substrate in assays. Specific concentrations of substrate used were 5 (WT, Y142F), 10 (Y142A), 20 (D15A, H144A) or 50 mM (D146A) GlcNAc.

**Equations**

**Eq. 1**

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

**Eq. 2**

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

**Eq. 3**

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

For determination of the steady-state parameters, deacetylase activity was measured at 6-8 different concentrations of GlcNAc (0 – 300 mM), and the parameters kₐₛ, Kₘ, and kₐₕ/Kₘ 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. For D146A, higher concentrations of GlcNAc (0 – 375 mM) were used.
Solvent viscosity assays were carried out using the microviscogens sucrose (0-35% (w/v)) and glycerol (0-35% (w/v)), and the macroviscogen Ficoll 400 (0-10% (w/v)) with a sub-saturating concentration (5 mM) or saturating concentration (100 mM) of GlcNAc. The $\eta_{rel}$ values for 0, 10, 20, 27.5, 32.5, and 35% sucrose are 1, 1.32, 1.88, 2.48, 3.06, and 3.42, respectively (24). The $\eta_{rel}$ values for 0, 10, 20, 30, and 35% glycerol are 1, 1.3, 1.7, 2.3, and 2.9, respectively (25). The $\eta_{rel}$ values for 0, 5, and 10% Ficoll 400 are 1, 2.2, and 4.5, respectively (26). Results for experiments examining the effect of solvent viscosity on H144A at 300 mM GlcNAc are included in Supplemental Data Figure S1.

Fluoride inhibition studies were carried out with 0-200 mM NaF added to the assay mixture. There was no effect on MshB activity observed in control experiments using an additional 200 mM NaCl in the assay mixture (Supplemental Data Figure S2). For solvent isotope effect experiments, initial rates at sub-saturating substrate concentrations - 5 (WT, Y142F), 10 (Y142A), 20 (D15A, H144A) or 50 mM (D146A) GlcNAc in H2O were compared to the initial rates in ~95% D2O. The pH values obtained for the D2O buffers using the pH meter readings were corrected by adding 0.4 to these values.

**Computational Studies**

Structural alignment of the six MshB monomers in available crystal structures (PDB 1Q74 and 1Q7T)(15,16) was carried out using the MatchMaker program in the UCSF Chimera package (22,27). Potential Y142 rotamers were evaluated using Chimera (22) with the Dunbrack backbone dependent rotamer library (28). This library contains six possible Tyr rotamers that are commonly observed in proteins. A model of MshB with Y142 positioned for a possible role in chemistry was prepared using Chimera by rotating Y142 into the location of a Tyr rotamer in the Dunbrack library.

**RESULTS**

**Mutations Decrease Catalytic Activity**

The crystal structure of Zn$^{2+}$-MshB (Figure 1b) reveals a zinc ion in the active site bound by the side chains of three protein ligands (His13, Asp16, His147) and one to two water molecules that is surrounded by side chains typically involved in acid-base catalysis and/or stabilization of oxanion intermediates (Asp15, His144, Asp146). Importantly, this structure reveals two potential acid-base catalysts that are in close proximity to the zinc-bound water molecules - Asp15 and His144; Asp15 is located ~2.8 Å from one zinc-bound water molecule, while His144 is ~3.4 Å away from the second zinc-bound water molecule. As a result of their close proximity to the catalytic zinc ion, these side chains are best positioned to serve as GBC/GAC in the chemical reaction. Crystal structures of a MshB-β-octyl-D-glucopyranoside (BOG) complex, which lack the catalytic metal ion, (Figure 1c) also indicate that these side chains are well positioned to interact with the substrate GlcNAc moiety (16). Additionally, the side chain of Asp146 is located near His13 and His144 (2.9 – 3.6 Å), suggesting a potential role for Asp146 in catalysis. On the basis of the crystal structure of the Zn$^{2+}$-MshB active site (15) and the pH-dependence of MshB deacetylase activity (14), the MshB-catalyzed reaction is proposed to proceed through either a single GABC or a GABC pair mechanism using the side chains of Asp15 and/or His144 (21).

To distinguish between these two possible mechanisms, we used a combination of mutagenesis and kinetic experiments. We prepared a series of MshB constructs wherein active site side chains (D15, H144, D146) were mutated to Ala using site-directed mutagenesis and the steady-state parameters for these constructs were determined using the substrate GlcNAc (Table 1, Supplemental Data Figure S3). Results from these experiments indicate that removal of the D15, H144, and D146 side chains lead to an overall decrease in catalytic activity suggesting that these side chains are important for maximal catalytic activity. Specifically, the D15A mutation leads to a modest (< 2-fold) increase in $K_M$, an ~80-fold decrease in $k_{cat}$, and a 222-fold decrease in $k_{cat}/K_M$. The H144A mutation leads to a modest increase in the value of $K_M$ to ~50 mM, but decreases the values of $k_{cat}$ and $k_{cat}/K_M$ 60- and 100-fold, respectively. The D146A mutant could not be saturated with [GlcNAc] of 375 mM; therefore, the steady-state parameters for this mutant are estimated values. The D146A mutation
results in a > 10-fold increase in $K_M$ and >100-fold decrease in $k_{cat}/K_M$.

**Solvent Viscosity Effects**

To aid in deciphering the rate-limiting step for MshB deacetylation, we examined the effect of solvent viscosity on the rate of MshB-catalyzed deacetylation. Solvent microviscosity slows the rates of steps that involve movement of small molecules, such as substrate binding and product release, as well as conformational changes in proteins (24-26,29-31). In contrast, rates for internal processes, such as chemistry, proceed independent of solvent microviscosity. To control for changes in activity that arise from non-specific interactions, we used multiple microviscogens (sucrose, glycerol), as well as the macroviscogen Ficoll 400. Macroviscogens alter solvent viscosity, but do not slow the rates of diffusion of small molecules and is used to control for changes in activity that arise from non-specific interactions (24,25,29-31).

We measured the effect of sucrose and glycerol (microviscogens), as well as Ficoll 400 (macroviscogen), on MshB activity. Results from experiments with WT MshB (Figure 2, Supplemental Data Table S1) indicate that both $k_{cat}$ and $k_{cat}/K_M$ are significantly slowed in the presence of the microviscogens sucrose and glycerol. For effects on $k_{cat}$, the slopes observed in the presence of glycerol and sucrose are 0.74 ± 0.13 and 0.62 ± 0.11, respectively. The finding that the plots for assays in the presence of glycerol and sucrose have comparable slopes suggests that the microviscogens are affecting the diffusion of small molecules and/or a conformational change in the protein, and not simply having non-specific effects on MshB (e.g., dielectric constant). For effects on $k_{cat}/K_M$, the slopes for assays in the presence of glycerol and sucrose are 1.72 ± 0.12 and 1.70 ± 0.28, respectively. Again, the finding that the plots for assays in the presence of glycerol and sucrose have comparable slopes suggests that the microviscogens are affecting the diffusion of small molecules and/or a conformational change in the protein, and not having non-specific effects on MshB. These initial plots of $k_{cat}$ or $k_{cat}/K_M$ vs. $\eta_{rel}$ show some deviation from linearity, and therefore, additional analyses were carried out on these data (below). As expected, the slopes of the plots examining the effect of Ficoll 400 on $k_{cat}$ (0.03 ± 0.006) and $k_{cat}/K_M$ (-0.086 ± 0.02) indicate that these parameters are unaffected by the macroviscogen Ficoll 400.

For rate-limiting steps where product release is associated with conformational changes in the protein, a plot of log $k_{cat}$ vs. log $\eta_{rel}$ is linear, while for rate-limiting steps where product release occurs in the absence of a structural rearrangement a plot of log $k_{cat}$ vs. $\eta_{rel}^2$ is linear (29,31). To gain insights into whether product release in MshB is dependent on a conformational change in the protein, we examined which of these plots better describe our data (Figures 2c and 2d, Supplemental Data Table S1). While both plots comparably describe data obtained using glycerol as the viscogen, the sucrose data are better described by the log $k_{cat}$ vs. log $\eta_{rel}$ plot, suggesting product release in MshB may be coupled to a conformational change in the protein. The finding that $k_{cat}/K_M$ is also better described by log $k_{cat}/K_M$ vs. log $\eta_{rel}$ (Supplemental Data Figure S4) may suggest that there is also a conformational change coupled with substrate binding to MshB.

We examined the effect of solvent viscosity on the activity of the D15A and H144A mutants (Figure 3, Supplemental Data Table S2) to determine if product release remains rate limiting for these catalytically impaired mutants. Once again we observe that $k_{cat}$ is significantly slowed in the presence of glycerol and sucrose. These data are clearly better described by a plot of log $k_{cat}$ vs. log $\eta_{rel}$ (Figure 3a) compared to a plot of log $k_{cat}$ vs. $\eta_{rel}^2$ (Figure 3b), suggesting that the rate-limiting step in these mutants is a conformational change that is coupled to product release. (Note: Since we are unable to reach substrate saturation with the D146A mutant, the effect of solvent viscosity could only be determined under $k_{cat}/K_M$ conditions. Supplemental Data Figure S4 and Table S3).

**Role of Metal-Water/Hydroxide**

We previously examined the ability of divalent metal ions to serve as cofactors for MshB and found that the overall activity follows the trend: Fe$^{2+}$ > Co$^{2+}$ > Zn$^{2+}$ > Mn$^{2+}$ > Ni$^{2+}$ (14). While these results confirm the importance of the metal cofactor for activity, the specific role of the metal ion in the MshB-catalyzed reaction has not
been elucidated. Therefore, we set out to probe the role of the metal ion in catalysis by MshB.

Fluoride is often used to probe the identity of the reactive nucleophile for metallohydrolases (32-35). Specifically, fluoride inhibits (uncompetitive) enzymes that utilize a metal-bound water or hydroxide as the reactive nucleophile. Therefore, we measured the MshB-catalyzed deacetylation of GlcNAc in the presence of various concentrations of NaF (0-200 mM). Results from these experiments (Supplemental Data Figure S5) show that NaF acts as an uncompetitive inhibitor of the deacetylation reaction consistent with the metal-water/hydroxide serving as the reactive nucleophile in the MshB-catalyzed reaction.

MshB activity (V/K) exhibits a bell-shaped dependence on pH with two ionizations having pKₐ values of ~7.3 (pKₐ₁) and 10.5 (pKₐ₂) (14). There is an increase in activity with increasing pH for the ionization described by pKₐ₁, while there is a decrease in activity with increasing pH for the ionization described by pKₐ₂. Since the metal-bound water could be reflected in one of these pKₐ values, we examined the pH-dependence of deacetylase activity for MshB reconstituted with various divalent metal ions. Results from these experiments are shown in Table 2. These results confirm MshB activity follows the trend Fe²⁺ > Co²⁺ > Zn²⁺ > Mn²⁺ > Ni²⁺. Furthermore, these findings indicate that the identity of the metal ion does not significantly alter pKₐ₁ and has only a modest effect on pKₐ₂ (0.6 pH units). These results rule out the metal-water as the source of pKₐ₁, while the small affect on pKₐ₂ (0.6 pH units) suggests that it is unlikely that this pKₐ reflects ionization of the metal-water either.

**Examination of Tyr142**

Since we could not identify the source of pKₐ₂ using metal substitution or our initial mutagenesis experiments (D15A, H144A and D146A), we examined the MshB crystal structures more closely for additional possible sources of this ionization. Importantly, the side chain of Y142 appears to be a dynamic side chain, moving several angstroms in the Zn²⁺-MshB and MshB-BOG complex structures. An overlay of the six MshB monomers (four Zn²⁺-MshB and two MshB-BOG) is shown in Figure 5a. The Y142 side chain is found in four different locations in the six monomers. The locations in red, orange, yellow and purple are observed in the four Zn²⁺-MshB monomers, while the blue and green locations are observed in MshB-BOG monomers. The locations of these side chains and rotation of Y142 in Chimera both indicate that the Y142 side chain is unobstructed and can move freely (360°) from being on the MshB surface/solvent exposed (purple) to various locations in the active site.

Additionally, we examined the Tyr rotamers using the Dunbrack database in Chimera (22,28). Tyr rotamers are most commonly observed in three general locations with two orientations of the aromatic ring at each location, yielding a total of six possible rotamers (Figure 5b). Examination of these locations reveals that two of the locations (rotamers 1-4) are observed in

\[ V/K \] parameter reflecting a conformational change coupled to product release, and therefore, does not provide information about the chemical step of the reaction (the pH profile under \( V \) conditions is included in Supplemental Data Figure S6). We examined the pH-dependence of the MshB Ala mutants (D15, H144, D146) and the results from these experiments are summarized in Table 3 and Figure 4. These results confirm that the side chains of D15, H144, and D146 are important for catalytic activity, as the rate at the pH-optimum is decreased ~180 to 530-fold. Importantly, we observe that pKₐ₁ is lost in the D15A mutant (Figure 4), suggesting that pKₐ₁ reflects ionization of D15 in the WT enzyme. While the H144A and D146A mutations decrease MshB activity, the pKₐ values observed for these mutants are the same as those observed for the WT protein, suggesting that neither H144 nor D146 is responsible for the ionizations observed in the WT protein.

**Mutations Alter pH-Dependence**

To gain further insights into the chemical mechanism of MshB, we probed the identities of the ionizations observed in the wild-type (WT) MshB pH-profile under sub-saturating concentrations of substrate (V/K). We chose to focus on the parameter V/K rather than V because V/K examines the reaction of free enzyme with free substrate through the first irreversible step, chemistry, and therefore, will provide information about the chemical mechanism of the enzyme. In contrast, results from solvent viscosity experiments (Figures 2 and 3) indicate that the
the Zn$^{2+}$-MshB structures, while the third location (rotamers 5 and 6) lies between the Y142 positions observed in the MshB-BOG structures. A model of MshB with Y142 in this third location is shown in Figure 5c (gray wire in Figure 5a). In this rotamer the hydroxyl group of Y142 is within hydrogen bonding distance to the metal-water, which would be capable of participating in catalysis. Therefore, we examined the effect of the Y142A mutant on catalytic activity.

The Y142A mutation significantly alters the steady-state parameters (Table 1). This mutation leads to a $\sim$2-fold decrease in $K_M$, 26-fold decrease in $k_{cat}$, and a 15-fold decrease in $k_{cat}/K_M$. To further examine the role of Y142 in catalysis, we examined the Y142F (sterics, hydrophobicity) and Y142Q (hydrogen bonding) mutants. The Y142F mutation leads to a 4-fold decrease in $K_M$, $\sim$80-fold decrease in $k_{cat}$, and a 20-fold decrease in $k_{cat}/K_M$, while the Y142Q mutation leads to a 2-fold decrease in $K_M$, $\sim$40-fold decrease in $k_{cat}$, and a 15-fold decrease in $k_{cat}/K_M$. Additionally, we observe that the ionization described by $pK_{a2}$ is no longer observed for Y142A or Y142F mutants (Figure 4). These results suggest that $pK_{a2}$ reflects ionization of Y142 in WT MshB.

Furthermore, we find that the solvent viscosity effect under $k_{cat}$ conditions described for MshB in WT and mutant (D15A, H144A) enzymes is lost in the Y142F mutant (Figures 2 and 3), while the solvent viscosity effect under $k_{cat}/K_M$ conditions described for WT is also significantly diminished in the Y142F mutant (Figure 2b, Supplemental Data Table S3, Figure S4).

Y142 Dynamics Modulate Access to Active Site

Mutation of Y142 to Ala, Phe or Gln leads to a $\leq$ 4-fold decrease in $K_M$, suggesting that Y142 plays a role in substrate binding. Since the location of Y142 varies from being on the surface of the protein (purple) to inside the active site, the Y142 side chain may modulate access to the active site. To probe if the side chain Y142 affects access to the enzyme active site, we examined the surfaces of each overlaid monomer (Figure 5a) with the two BOG molecules (Figure 1c) as a model for GlcNAc-Ins binding. The results are shown in Figure 6 and suggest that the structural changes that occur to MshB upon movement of Y142 appear to alter access to the active site. Panels A and C represent the same structures from two different viewpoints with the side chain of Y142 shown in dark gray. In the four Zn$^{2+}$-MshB monomers (red, orange, yellow, purple) both sugar molecules are visible indicating that these Tyr conformations would allow for substrate binding and product release. In the two MshB-BOG monomers (blue, green) the sugar molecules become obscured indicating that the movement of substrate and product in/out of the active site would be hindered in these Tyr conformations. Panel B is a representation of the viewpoint depicted in Panel A, wherein the side chain of Y142 has been truncated to Ala (dark gray) in attempts to visualize the changes that occur upon the Y142A mutation. Similarly, Panel D is a representation of the viewpoint depicted in Panel C, wherein the side chain of Y142 has been truncated to Ala (dark gray). The information in Panels B and D predict that there should better access to the active site in the Y142A mutant, as the sugar molecules become more visible.

Solvent Isotope Effects

Since the proposed reactions involve GABC, we examined if a solvent isotope effect is observed for the MshB-catalyzed reaction. Solvent isotope effects of 2-4 are typically observed for reactions that proceed through GBC, while inverse solvent isotope effects are observed for reactions that proceed through GAC (36). We measured the pH-dependence of the solvent isotope effect for the WT protein under V/K conditions (Figure 7a, Table 4). Results from these experiments reveal a small normal solvent isotope effect on V/K in WT MshB. This finding suggests that either 1.) the chemistry step reflected in V/K has contributions from both GAC and GBC, or 2.) that proton transfer is not a significant rate-determining step under these conditions.

To aid in deciphering between these two possibilities and to gain additional insights into the chemical mechanism, we also measured the pH-dependence of the solvent isotope effect for the MshB mutants D15A, Y142F, and H144A under V/K conditions. The results from these experiments are shown in Figure 7 and Table 4. We observe a slight inverse solvent isotope effect of 0.8 for the D15A mutant (Figure 7b), while there is no solvent isotope effect for the Y142F...
mutant (Figure 7d). The most striking observation is the large solvent isotope effect for the H144A mutant (Figure 7c) of ~5. There is no significant difference in the pK_a values observed between H_2O and D_2O for any of the mutants examined.

**DISCUSSION**

*MshB Uses Key Protein Side Chains and a Metal-Water/Hydroxide as the Reactive Nucleophile*

Results from mutagenesis studies (Tables 1 and 3) indicate that active site side chains D15, Y142, H144 and D146 are all important for catalytic activity. The locations of D15 and H144 (Figures 1b and 1c) suggest that these side chains may function as GABC in the chemical mechanism, while the location of Y142 in the model (Figure 5c) suggests that this side chain may play a role in polarization of substrate and/or stabilization of the oxyanion intermediate. The location of D146 suggests that this side chain does not directly participate in the chemical mechanism, but plays an indirect role via interaction(s) with the side chain(s) of His13 and/or His144 (Figures 1b and 1c). Titration of apo-D146A with Zn^{2+} (Supplemental Data Figure S7) suggests that the loss of activity observed for this mutant cannot be attributed to decreased binding of the catalytic Zn^{2+} ion via interaction with the zinc ligand H13 under the reaction conditions. Previous results have shown that the activity of MshB is dependent on the identity of the catalytic metal ion (14). Herein we demonstrate that NaF is an uncompetitive inhibitor of MshB (Supplemental Data Figure S5). These results are consistent with MshB using a metal-bound water/hydroxide as the reactive nucleophile in the reaction.

*Parameter k_cat Reflects a Conformational Change Coupled to Product Release*

Results from solvent viscosity experiments indicate the parameter k_cat/K_M is inhibited by microviscogens (sucrose, glycerol), not macroviscogens (Ficoll 400). This is expected given that the parameter k_cat/K_M reflects substrate binding through the first irreversible step (i.e., chemistry) and suggests that k_cat/K_M is partially limited by substrate association in WT MshB. The finding that the effect of solvent viscosity on k_cat/K_M is diminished in the Y142F mutant (Figure 2b, Supplemental Data Table S3) may suggest that the Y142 side chain is involved in the conformational change coupled to substrate binding, or that chemistry becomes more rate-limiting under k_cat/K_M conditions in catalytically slow mutants.

Results from solvent viscosity experiments also indicate that the parameter k_cat is inhibited by the microviscogens glycerol and sucrose, suggesting k_cat reflects a step that involves the diffusion of small molecules (i.e., substrate binding, product release) and/or a conformational change in MshB. Since both the WT and mutant data are better described by plots of log k_cat vs. log \eta_{rel} compared to plots of log k_cat vs. \eta_{rel}^2, the rate-limiting step for MshB is likely a conformational change that is associated with product release or substrate binding. The finding that the value of k_cat is similar with the substrates GlcNAc-Ins (0.49 s^-1) and GlcNAc (0.77 s^-1), while K_M values for these substrates vary significantly (340 mM and 38 mM, respectively), is consistent with the hypothesis that k_cat reflects a conformational change that is associated with product release in WT MshB rather than simple product dissociation (12,23).

The findings that: 1.) mutation of Y142 (Ala, Gln, Phe) results in a 15-20-fold decrease in k_cat, 2.) the Y142F mutant is the only MshB variant examined where the value of the parameter k_cat is unaffected by changes in solvent viscosity, 3.) the location of the Y142 side chain appears to be dynamic in available crystal structures, and 4.) the value of k_cat is identical for the D15A and Y142F mutants suggest that the Y142 side chain, likely the hydroxyl group, is involved in the conformational change that is associated with product release. The slopes for log k_cat vs. log \eta_{rel} plots range from 0 to 1 depending on degree of coupling of active site to solvent (25). The finding that the slopes for WT MshB obtained in the presence of glycerol and sucrose are 0.72 ± 0.09 and 0.81 ± 0.06, respectively, suggests that there is significant coupling between the dynamics of MshB and solvent molecules. This is expected given the role of Y142 in modulating the dynamics of MshB and its observed location(s) in the enzyme (Figures 5 and 6). Furthermore, results from kinetics data support a role for Y142 in product release as the parameter k_cat is
significantly decreased upon mutation of Y142 (Table 1). Specifically, we observe that the value of $K_{a1}$ for the Y142 mutants follows the trend Ala > Gln > Phe for substitution at position 142, suggesting that the product release becomes slower with increasing hydrophobicity of the side chain (Supplemental Data Figure S8; correlation plots for $K_{a1}$ also provided). It is likely that the rate acceleration with Tyr at this position arises from a combination of factors (i.e., hydrophobicity, hydrogen bonding interactions, length of side chain).

**Ionization of Asp15 and Tyr142 are Important for Maximal Activity**

Interestingly, $pK_{a1}$ is not observed in the D15A mutant (Figure 4) suggesting that this $pK_a$ reflects ionization of the D15 side chain in WT MshB. Since the ionization described by $pK_{a1}$ leads to an increase in activity with increasing pH, D15 most likely functions as a GBC in the reaction to deprotonate the metal-bound water, activating it for attack of the carbonyl group on substrate. Structural data available support this mechanism as the side chain of D15 shares a hydrogen bond to one of the metal-waters in the MshB crystal structure (Figure 1b). The magnitude of the decrease in activity observed for the D15A mutant (~530-fold) is smaller than that observed for side chains that are bifunctional GABC ($10^3$-$10^5$-fold) (37-39), and consequently, this side chain is likely part of a GABC pair that catalyzes the deacetylation reaction. The $pK_a$ of D15 is likely elevated due to the environment of the active site, which includes an adjacent D16 side chain.

Results from pH studies also reveal that the ionization described by $pK_{a2}$ is lost in the Y142F and Y142A mutants (Figure 4), suggesting that the side chain of Y142 is responsible for $pK_{a2}$ in WT MshB. The ionization described by $pK_{a2}$ leads to a decrease in activity with increasing pH, indicating that the Tyr must remain protonated for maximal activity. This suggests two possible roles for Y142 in the chemical reaction: 1.) GAC to protonate the amine leaving group, or 2.) polarize the carbonyl substrate/stabilize the oxyanion intermediate. Due to the magnitude of the decrease in activity at the pH optima ($V/K$) compared to the H144A mutant (74- vs. 179-fold), the distance between the Y142 hydroxyl and metal-water (2.15 Å) in the catalytic model depicted in Figure 5c, and the orientation of Y142 relative to the presumed substrate binding site (Figures 1c and 6) it seems most likely that Y142 functions in the latter role to polarize the carbonyl group on substrate/stabilize the oxyanion intermediate. The modest change that is observed for $pK_{a2}$ upon substitution of the active site metal ion (Table 2) may also be explained by this ionization reflecting Y142 since this side chain is proposed to hydrogen bond to the metal-water, and therefore, would be sensitive to the identity of the bound metal ion. The assignment of $pK_{a2}$ reflecting the ionization of Y142 is preferred over assignment of $pK_{a2}$ as the metal-water because 1.) the $pK_a$ is lost in the Y142A/F mutants (compared to a modest shift in $pK_{a2}$ observed in the metal substitution experiments), and 2.) it seems unlikely that substitution of Y142 with Phe and Ala would both markedly increase the $pK_a$ of the metal-water by several pH units given the differences in hydrophobicity between the active sites containing Phe vs. Ala at this position.

The decrease in activity of H144A mutant (~180-fold) and location of the side chain (Figures 1b and 1c) are consistent with H144 serving as the GAC in the reaction working in concert with D15 as part of a GABC-pair. The magnitude of the activity decreases for the D15A (530-fold) and H144A (~180-fold) mutants are more consistent with the loss of a single GBC or GAC that function as part of a GABC pair rather than the loss of residues that function as bifunctional GABC ($10^2$-$10^3$-fold) (37-39). Additionally, results from solvent isotope effect experiments are consistent with a mechanism that uses a GABC pair. Specifically, these results suggest that there is no significant solvent isotope effect observed in the WT protein because it includes contributions from both GAC and GBC. In the D15A mutant where the proposed GBC has been removed, there is a small inverse solvent isotope effect (~0.8) observed that is consistent with a reaction that proceeds via GAC. In the H144A mutant where the proposed GAC has been removed, there is a large solvent isotope effect (~5) observed that is consistent with a reaction that proceeds via GBC. Taken together, these results support a model in which D15 and H144 function as a GABC pair in the WT MshB-catalyzed reaction.
**Chemical Mechanism**

Together these data suggest that MshB proceeds via the chemical mechanism shown in Figure 8. In this mechanism, the carbonyl group on substrate replaces one of the metal-waters. Upon binding, the catalytic metal ion and the side chain of Y142 polarize the substrate carbonyl group. The side chain of D15 functions as a GBC to activate the metal-water for attack on the carbonyl substrate. The catalytic metal ion and Y142 stabilize the resulting oxyanion tetrahedral intermediate. Finally, the side chain of H144 functions as a GAC to facilitate breakdown of the tetrahedral intermediate. This proposed mechanism using a GABC pair is similar to the mechanism used by the metal-dependent deacetylase LpxC and unlike prototypical metalloprotease carboxypeptidase A and the histone deacetylase (HDAC) enzymes which use a single bifunctional GABC for catalysis (21).

**Dynamics of Y142 Play Critical Role in Catalysis**

The changes in $K_M$ that are observed in the Y142 mutants, solvent viscosity effects, and the overlay of structures with BOG molecules (Figure 6) are consistent with Y142 playing a role in substrate binding and product release, while the activity data indicate that Y142 plays a role in the chemical mechanism. These different roles for MshB in catalysis can be described using the structures in Figure 6. We hypothesize that when Y142 is located in the red and orange positions, the active site is open and allows for substrate binding. Movement of Y142 (counterclockwise) towards the catalytic metal ion blocks access to the active site and allows for participation of Y142 in catalysis (model lies between blue and green positions). Following participation in chemistry, Y142 continues moving counterclockwise to the purple and then red locations allowing for product release. Results from solvent viscosity experiments are consistent with a role for Y142 assisting in substrate binding and product release, while effects on $k_{cat}/K_M$ and $V/K_M$ support a role for Y142 in chemistry. Together our findings suggest that the dynamic Y142 plays a critical role in catalysis by modulating substrate binding, chemistry, and product release.

**SUPPLEMENTAL DATA**

Kinetic plots (solvent viscosity effects, effect of NaCl on MshB activity, Michaelis-Menten plots for MshB mutants, NaF inhibition, and solvent isotope effects under $V$ conditions, titration of D146A with Zn$^{2+}$) and tables of solvent viscosity data are provided as supplemental data.

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FOOTNOTES

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Keywords: MshB; metal-dependent deacetylase; metallohydrolase; mechanism; iron; zinc; mycothiol; GABC pair

Abbreviations: MSH, mycothiol; MshB, N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-α-D-glucopyranoside deacetylase; MCA, mycothiol-conjugate amidase; GlcNAc-Ins, N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-α-D-glucopyranoside; GlcN-Ins, 1-D-myo-inositol-2-amino-2-deoxy-α-D-glucopyranoside; GABC, general acid-base catalysis; GBC, general base catalyst; GAC, general acid catalyst; IMAC, immobilized metal ion affinity chromatography; PDF, peptide deformylase; LuxS, S-ribosylhomocysteinase; LpxC, UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglicosamine deacetylase; HDAC8, histone deacetylase 8; TCEP, tris(carboxyethyl)phosphine; 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; BOG, β-octyl-D-glucopyranoside; WT, wild-type.

FIGURE LEGENDS

Figure 1: (A) Reaction catalyzed by MshB (B) Active site of MshB (PDB 1Q74) containing a catalytic zinc ion. Only one of the four Zn²⁺-MshB monomers is shown. (C) Active site of MshB (PDB 1Q7T) containing bound β-octyl-D-glucopyranoside (BOG). The structures of the two MshB-BOG monomers were overlaid revealing two different locations for glucose binding to MshB, BOG1 and BOG2. The octyl chains of the active site BOG molecules are not observed in either monomer.

Figure 2: Effect of Solvent Viscosity on (A) $k_{cat}$ and (B) $k_{cat}/K_M$. The effects of the microviscogens sucrose (0-35% (w/v)) and glycerol (0-35% (w/v)) on WT MshB are depicted as (○) and (●), respectively, while the macroviscogen Ficoll 400 (0-10% (w/v)) is denoted as (□). Data for the Y142F mutant in the presence of sucrose is depicted by (∇). Plots (C) and (D) are used to determine if there is a conformational change associated with product release and/or substrate binding. Apo-MshB was incubated with stoichiometric Zn²⁺. After 30 minutes, the enzyme was diluted into assay buffer (50 mM HEPES, 1 mM TCEP, 50 mM NaCl pH 7.5) containing substrate and the initial rates for the deacetylation of a sub-saturating (5 mM) or saturating (100 mM) concentration of GlcNAc were measured as described under “Materials and Methods”. The slopes are provided in Supplemental Data Table S1.

Figure 3: Effect of Solvent Viscosity on $k_{cat}$ of MshB mutants. The effects of the microviscogens sucrose (0-35% (w/v)) and glycerol (0-35% (w/v)) on WT MshB are depicted as open and closed symbols, respectively. MshB mutants examined are: D15A (circles), H144A (squares), and Y142F (triangles). Plots (A) and (B) are used to determine if a conformational change is associated with product release/substrate binding. Apo-MshB was incubated with stoichiometric Zn²⁺. After 30 minutes, the enzyme was diluted into assay buffer (50 mM HEPES, 1 mM TCEP, 50 mM NaCl pH 7.5) containing substrate and the initial rates for the deacetylation at saturating concentrations (100 mM) of GlcNAc were measured as described under “Materials and Methods”. The slopes of the fits are provided in...
Supplemental Data Table S2. The effect of glycerol on H144A activity using 300 mM GlcNAc is included in supplemental Figure S1.

**Figure 4:** pH rate profiles. The effect of pH on the deacetylation of GlcNAc by MshB under sub-saturating substrate concentrations (V/K). The pH dependence of the MshB-catalyzed reaction is shown for WT (○), D15A (□), Y142A (△), Y142F (▲), H144A (▼), and D146A (◇). Assays were measured at 30°C with sub-saturating (5-50 mM) concentrations of GlcNAc as described under “Materials and Methods”. The pKₐ values were determined by fitting the equation including two ionizations (Eq. 1) or one ionization (Eq. 2 or 3) and are shown in Table 3.

**Figure 5:** MshB active site. (A) Overlay of the six MshB monomers. The locations of the active site side chains (H13, D15, D16, H144, D146, H147) do not vary significantly, while the location of the Y142 side chain (rainbow colored) varies in the structures. A model of the Zn²⁺-MshB active site with Y142 rotated into a catalytic location is represented by the gray wire. (B) Potential Tyr rotamers. The six Tyr rotamers (1-6) from the Dunbrack library are displayed as wires. The six rotamers fall into three general locations with two orientations of the phenyl ring at each location. The location of the purple side chain in panel A is represented by the gray stick. (C) Zn²⁺-MshB active site with Y142 rotated into a catalytic location (position of rotamer 5).

**Figure 6:** MshB active site accessibility. The panels show the surfaces of the six MshB monomers from the overlaid structures in Figure 6a for two different viewpoints. The surface colors correspond to locations of Y142 shown in Figure 6a and the dark gray is the Y142 side chain. The substrate GlcNAc-Ins is presumed to bind in the location of the two BOG molecules (colored in red and light gray) that are observed in the MshB-BOG structures (Figure 1c). A) Viewpoint 1 with the Y142 side chain shown in dark gray. B) Viewpoint 1 where the Y142 side chain was truncated to Ala (dark gray). C) Viewpoint 2 with the Y142 side chain shown in dark gray. D) Viewpoint 2 where the Y142 side chain was truncated to Ala (dark gray).

**Figure 7:** Solvent isotope effect of MshB variants. The solvent isotope for MshB was measured in H₂O (open symbols) and 95% D₂O (closed symbols) at 30°C. Solvent isotope effects were measured under sub-saturating substrate concentrations of GlcNAc. The values for V/K were measured using 5 or 20 mM GlcNAc as described under “Materials and Methods”. (A) WT MshB in H₂O (○) and 95% D₂O (●). (B) D15A in H₂O (□) and 95% D₂O (■). (C) H144A in H₂O (▼) and 95% D₂O (▲). (D) Y142F in H₂O (△) and 95% D₂O (▲). The pKₐ values were determined by fitting an equation including one or two ionizations (Eq. 1-3) to these data and are shown in Table 4. Fits for H₂O data are represented with dashed lines and D₂O data are represented with solid lines.

**Figure 8:** Proposed mechanism for MshB.
### Table 1. Steady-state kinetic parameters of MshB mutants

| MshB<sup>a,b</sup> | $K_M$ (mM) | $k_{cat}$ (min<sup>-1</sup>) | $k_{cat}/K_M$ (M<sup>-1</sup>s<sup>-1</sup>) | % WT activity |
|-------------------|-----------|-----------------|------------------|----------------|
| WT<sup>c</sup>    | 38 ± 4    | 46 ± 2          | 20 ± 1           | --             |
| D15A              | 52 ± 11   | 0.29 ± 0.02     | 0.09 ± 0.01      | 0.5            |
| Y142A             | 22 ± 2    | 1.8 ± 0.05      | 1.36 ± 0.10      | 6.8            |
| Y142F             | 9 ± 0.5   | 0.57 ± 0.01     | 1.05 ± 0.05      | 5.3            |
| Y142Q             | 17 ± 2    | 1.2 ± 0.04      | 1.17 ± 0.11      | 5.9            |
| H144A             | 57 ± 8    | 0.69 ± 0.04     | 0.20 ± 0.02      | 1.0            |
| D146A             | > 400     | > 2             | ~0.08<sup>d</sup> | < 0.4          |

<sup>a</sup> apo-MshB was incubated with stoichiometric Zn$^{2+}$ for 30 min prior to activity measurement; <sup>b</sup> Substrate used GlcNAc; <sup>c</sup> Data adapted from (14); <sup>d</sup> Estimated from slope of initial linear region on v vs. [S] plot.
### Table 2. Effect of metal ions on pH-dependence of wild-type MshB

| Me^{2+}-MshB<sup>a,b</sup> | pK<sub>a1</sub> | pK<sub>a2</sub> | V/K (M<sup>-1</sup> s<sup>-1</sup>) at pH optimum |
|-----------------------------|---------------|---------------|----------------------------------|
| Zn<sup>c</sup>             | 7.4 ± 0.07    | 10.5 ± 0.06   | 48 ± 1.5                         |
| Co                          | 7.2 ± 0.09    | 11.0 ± 0.09   | 56 ± 2.3                         |
| Fe                          | 7.2 ± 0.09    | 10.5 ± 0.08   | 63 ± 2.3                         |
| Ni                          | 7.1 ± 0.03    | 11.1 ± 0.03   | 7.3 ± 0.1                         |
| Mn                          | 7.2 ± 0.12    | 11.0 ± 0.12   | 8.0 ± 0.4                         |

<sup>a</sup> apo-MshB was incubated with stoichiometric metal for 30 min prior to activity measurement;  
<sup>b</sup> Substrate 5 mM GlcNAc;  
<sup>c</sup> Data adapted from (14)
Table 3. pH-dependence of Zn\(^{2+}\)-MshB variants

| MshB\(^{a,b}\) | pK\(_{a1}\) ± | pK\(_{a2}\) ± | \(V/K\) (M\(^{-1}\) s\(^{-1}\)) at pH optimum | % WT activity |
|-----------------|--------------|--------------|--------------------------------|--------------|
| WT\(^c\)        | 7.4 ± 0.07   | 10.5 ± 0.06  | 48.2 ± 1.5                     | _            |
| D15A            | _            | 10.5 ± 0.13  | 0.091 ± 0.003                  | 0.2          |
| Y142A           | 7.1 ± 0.10   | _            | 0.96 ± 0.03                    | 2.0          |
| Y142F           | 7.0 ± 0.05   | _            | 0.65 ± 0.01                    | 1.3          |
| H144A           | 7.1 ± 0.10   | 10.5 ± 0.09  | 0.27 ± 0.01                    | 0.6          |
| D146A           | 7.2 ± 0.10   | 10.4 ± 0.10  | 0.18 ± 0.01                    | 0.4          |

\(^a\) apo-MshB was incubated with stoichiometric metal for 30 min prior to activity measurement; \(^b\) Substrate 5 (WT, Y142F), 10 (Y142A), 20 (D15A, H144A) or 50 mM (D146A) GlcNAc; \(^c\) Data adapted from (14)
Table 4. Solvent isotope effects of Zn\textsuperscript{2+}-MshB variants

| MshB\textsuperscript{a,b} | pK\textsubscript{a1} | pK\textsubscript{a2} | V/K\textsubscript{H2O}/V/K\textsubscript{D2O} |
|------------------------|-----------------|-----------------|--------------------------|
| WT\textsuperscript{c} (H\textsubscript{2}O) | 7.4 ± 0.1 | 10.5 ± 0.1 | 1.2 |
| (D\textsubscript{2}O) | 7.6 ± 0.1 | 10.6 ± 0.1 | |
| D15A (H\textsubscript{2}O) | -- | 10.4 ± 0.3 | 0.8 |
| (D\textsubscript{2}O) | -- | 10.3 ± 0.3 | |
| Y142F (H\textsubscript{2}O) | 7.1 ± 0.05 | -- | 1.0 |
| (D\textsubscript{2}O) | 7.3 ± 0.05 | -- | |
| H144A (H\textsubscript{2}O) | 7.1 ± 0.1 | 10.5 ± 0.1 | 5.4 |
| (D\textsubscript{2}O) | 7.4 ± 0.4 | 10.9 ± 0.4 | |

\textsuperscript{a}apo-MshB was incubated with stoichiometric metal for 30 min prior to activity measurement; \textsuperscript{b} Substrate 5 (WT, Y142F), 10 (Y142A), or 20 mM (D15A, H144A) GlcNAc; \textsuperscript{c} Data adapted from (14)
FIGURES

Figure 1:

A

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

B

C

\[
\text{His144} \quad 3.44\text{Å} \quad \text{Asp15} \quad 2.83\text{Å} \quad \text{Asp146} \quad \text{His147}
\]

\[
\text{BOG2} \quad \text{BOG1} \quad \text{His13} \quad \text{His144} \quad \text{Asp146} \quad \text{His147}
\]
Figure 2:
Figure 3:

A

B
Figure 4:
Figure 5:
Figure 7:
Figure 8:
Examination Of the mechanism Of N-Acetyl-1-D-Myo-Inosityl-2-Amino-2-Deoxy-α-D-Glucopyranoside deacetylase (MshB) reveals an unexpected role for a dynamic tyrosine

Xinyi Huang and Marcy Hernick

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