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Molecular Determinants of *N*-Acetylglucosamine Recognition and Turnover by *N*-Acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside Deacetylase (MshB)

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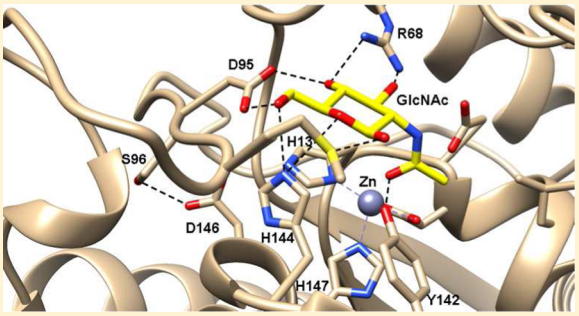
Supporting Information

ABSTRACT: Actinobacteria such as *Mycobacterium tuberculosis* use the unique thiol mycothiol (MSH) as their primary reducing agent and in the detoxification of xenobiotics. *N*-Acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase (MshB) is the metal-dependent deacetylase that catalyzes the deacetylation of *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside, the committed step in MSH biosynthesis. We previously used docking studies to identify specific side chains that may contribute as molecular determinants of MshB substrate specificity [Huang, X., and Hernick, M. (2014) *Biopolymers 101*, 406−417]. Herein, we probe the molecular basis of *N*-acetylglucosamine (GlcNAc) recognition and turnover by MshB using a combination of site-directed mutagenesis and kinetic studies (mutants examined, L19A, E47A, R68A, D95A, M98A, D146N, and F216A). Results from these studies indicate that MshB is unable to catalyze the turnover of GlcNAc upon loss of the Arg68 or Asp95 side chains, consistent with the proposal that these side chains make critical hydrogen bonding interactions with substrate. The activity of the D146N mutant is ∼10-fold higher than that of the D146A mutant, suggesting that the ability to accept a hydrogen bond at this position contributes to GlcNAc substrate specificity. Because there does not appear to be a direct contact between Asp146 and substrate, this effect is likely mediated via positioning of other catalytically important residues. Finally, we probed side chains located on mobile loops and in a hydrophobic cavity and identified two additional side chains (Met98 and Glu47) that contribute to GlcNAc recognition and turnover by MshB. Together, results from these studies confirm some of the molecular determinants of GlcNAc substrate specificity by MshB, which should aid the development of MshB inhibitors.



Actinobacteria make up a group of Gram-positive bacteria with a high G+C content and include the organism *Mycobacterium tuberculosis* that is responsible for the disease tuberculosis (TB). In spite of the attention that TB has received, it remains a global challenge therapeutically. In 2010, there were 8.7 million incident cases and 1.4 million deaths attributed to TB worldwide.[1] Consequently, there continues to be a need for new therapeutic agents for the treatment of TB, including drugs that can be used for the treatment of drug-resistant and latent TB infections.

One potential source of drug targets for the treatment of TB is mycothiol [MSH (Figure 1)]. MSH is a low-molecular weight thiol that serves roles analogous to those of glutathione in mycobacteria, functioning as both the primary reducing agent of this organism and a cofactor for the detoxification of xenobiotics.[2−6] The metal-dependent deacetylase *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase (MshB) catalyzes the fourth, and rate-limiting,[7] step in the MSH biosynthetic pathway, the deacetylation of *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins), to form 1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside (GlcN-Ins) and acetate (Figure 1).[2−6] In light of its role in MSH biosynthesis, and because there are no homologous enzymes in humans, MshB is considered an attractive drug target. The development of MshB inhibitors will be facilitated by information about the molecular determinants of ligand recognition and turnover by MshB that can be exploited for optimization of inhibitor potency and specificity. Our understanding of MshB has been aided by each of the three crystal structures of MshB that have been determined to date: Zn-MshB in the absence of bound ligand [Zn-MshB, Protein Data Bank (PDB) entry 1Q74],[8] as well as MshB complexes with β-octyl glucoside (BOG-MshB, PDB entry 1Q7T)[9] and bound acetate and glycerol (Act-MshB, PDB entry 4EWL).[10] These structures not only offer insights into different enzyme−ligand interactions but also capture MshB with different side chain conformations.

In addition to its natural substrate GlcNAc-Ins, MshB is also able to catalyze the hydrolysis of various deacetylase [i.e.,

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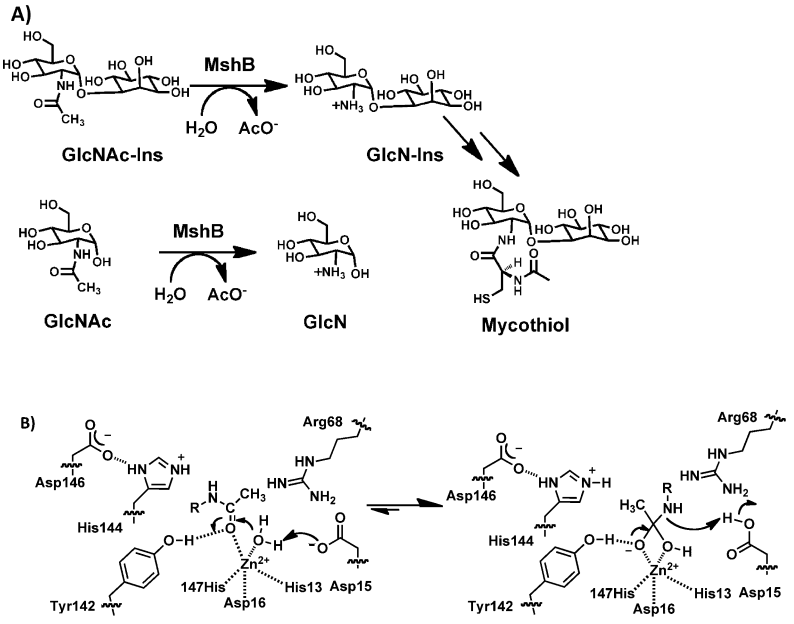

Figure 1. (A) Reactions catalyzed by MshB. (B) Proposed mechanism for MshB.

GlcNAc-Ins, *N*-acetylglucosamine (GlcNAc)] and amidase [i.e., bimane derivatives of desacetylmycothiol (CySmB-GlcN-Ins) and MSH (MSmB), formylated CysmB-GlcN-Ins (fCySmB-GlcN-Ins)] substrates (Figure 2).[11] We recently reported

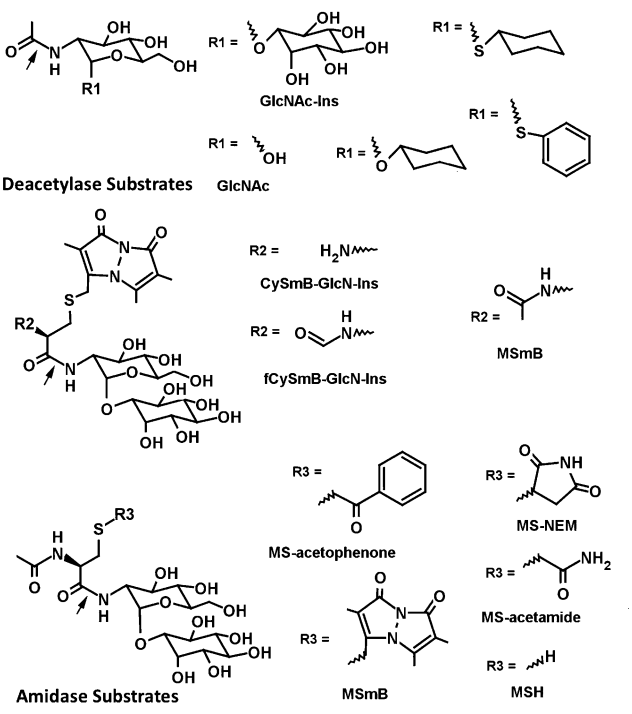

Figure 2. Structures of known substrates for MshB.

results from docking studies with a series of known MshB deacetylase and amidase substrates that identified a series of side chains that may play roles in ligand recognition and turnover by MshB (Figure 3) that can be examined using biochemical and biophysical approaches.[12] These studies were conducted using Act-MshB as the receptor, because this is the only structure that captures the Tyr142 side chain in the position required to enhance the rate of chemistry.

Herein, we examine the molecular basis for substrate recognition and turnover of the smaller GlcNAc substrate by MshB using a combination of site-directed mutagenesis and kinetic studies. Specifically, these experiments were designed to determine if the interactions predicted from docking studies could be verified through biochemical experiments. Docking studies with GlcNAc and MshB predicted this substrate is recognized via extensive hydrogen bonding interactions with the active site side chains of Arg68, Asp95, Tyr142, and His144 (Figure 3).[12] We have previously confirmed the importance of Tyr142 and His144 in GlcNAc recognition and turnover by MshB, and here we report results from kinetic studies of the R68A and D95A mutants that confirm the importance of these side chains in GlcNAc recognition and turnover by MshB. Additionally, results from these kinetic studies also suggest a role for Met98 in GlcNAc recognition and turnover by MshB, while loss of the Glu47 side chain leads to an enhancement of GlcNAc turnover by MshB. Together, these results build upon our current understanding of MshB and specifically offer new insights into the molecular basis of GlcNAc recognition and turnover by MshB, which may be useful for the development of MshB inhibitors.

MATERIALS AND METHODS

General Procedures. Primers for mutagenesis were purchased from Integrated DNA Technologies. DNA sequencing was performed at the Virginia Bioinformatics Institute DNA Sequencing Facility (Virginia Tech). All chemicals were purchased from Gold Biotechnology, Sigma-Aldrich, and ThermoFisher Scientific. Milli-Q water was used for the preparation of all solutions. For kinetic experiments, solutions were prepared using reagents lacking extraneous metal ions and/or were treated with Chelex (Bio-Rad) and stored in "metal-free" plasticware. Molecular graphic images were prepared using the UCSF Chimera package.[13]

Protein Expression and Purification. The previously reported plasmid encoding the *Mycobacterium smegmatis* MshB gene with an N-terminal His-maltose-binding protein (MBP) tag was used as the template for the preparation of mutant

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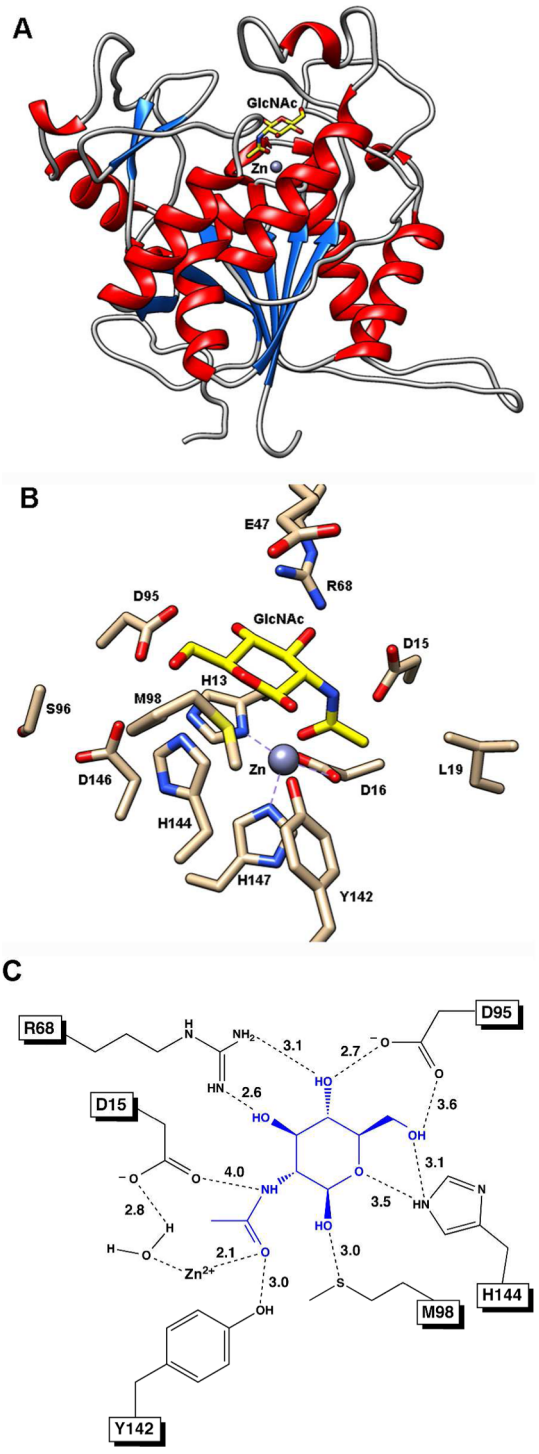

Figure 3. Model of the GlcNAc-MshB complex adapted from ref 12. (A) Overall structure of the MshB-GlcNAc complex. The bound GlcNAc (yellow) substrate is coordinated to the catalytic zinc ion (gray) through the carbonyl group. (B) Close-up of the GlcNAc-binding site. The bound GlcNAc (yellow) substrate is coordinated to the catalytic zinc ion (gray) through the carbonyl group. (C) Two-dimensional diagram illustrating the atom−atom distances (angstroms) of potential hydrogen bonding interactions observed in the docked GlcNAc-MshB complex.

plasmids using the QuikChange Lightning site-directed mutagenesis kit (Stratagene).[14] All of the mutants examined in these studies were sufficiently stable to allow for protein expression and purification under the conditions used for wild-type (WT) MshB and yielded soluble protein expression levels comparable to that of the WT protein (except the D146N mutant had a noticeably lower level of soluble protein expression compared to that of WT MshB) as determined via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). These findings are consistent with the predicted stabilities ($\Delta\Delta G$ values of ±2 kcal/mol) of the mutants calculated using I-Mutant 2.0, I-Mutant 3.0, and NeEMO (see Supporting Information Table S1).[17−19] I-Mutant and NeEMO were selected for these calculations since they are two of the more reliable programs for predicting the protein stability of mutants.[17,20] Predictions for the change in folding rates for each of the mutants was calculated using Folding RaCe (see Table S1 of the Supporting Information).[21] With the exception of D146A, all of the mutants are predicted to fold faster than WT MshB (log change folding rate from −0.07 to −1.55 s^{-1}).

MshB variants were expressed and purified according to published procedures.[14−16] Briefly, following expression, cells were resuspended in buffer A [30 mM HEPES, 150 mM NaCl, and 1 mM TCEP (pH 7.5)] and lysed, and the MshB variants were purified using Co-immobilized metal ion affinity chromatography (IMAC) [50 mL of chelating Sepharose (GE Healthcare) charged with $CoCl_2$] at 4 °C with an imidazole step gradient (buffer A and 0.5 to 300 mM imidazole). His-tobacco etch virus (TEV) protease (300 μg/mL) was added to the purified His-MBP-MshB variants from the initial Co-IMAC column prior to dialysis (Snake-skin tubing, molecular weight cutoff of 10000) against 2 × 4 L of buffer A at 4 °C for removal of the His-MBP tag. Cleaved MshB variants were purified from His-MBP-MshB and His-TEV using a second Co-IMAC column wherein the cleaved MshB variants elute in the flow-through. Fractions containing MshB (via 12% SDS−PAGE) were combined, concentrated, dialyzed against 2 × 4 L of buffer A (molecular weight cutoff of 10000), flash-frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined using the Bradford assay (Sigma).

Prior to activity measurements, all metal ions were removed from the purified protein and the resulting apoprotein was reconstituted with a stoichiometric concentration of zinc. Apo-MshB was prepared by incubation with 10 mM HEPES, 20 mM dipicolinic acid, and 250 μM EDTA (pH 7.5), on ice, as previously described.[14] Apo-MshB samples contained ≤10% metal/protein as determined using an ICS-3000 ion chromatography system (Dionex). Prior to activity measurements, apo-MshB (≤10 μM) was reconstituted with a stoichiometric concentration of $ZnSO_4$ as previously described.[14]

MshB Deacetylase Activity. A fluorescamine (FSA)-based assay was used to measure MshB deacetylase activity with the substrate GlcNAc (Sigma).[15] Although it is presumed that the GlcNAc substrate (K_M = 38 mM) has a decreased affinity for MshB compared with that of the natural GlcNAc-Ins substrate (K_M = 340 μM), the GlcNAc moiety undergoing the chemical transformation is preserved in the smaller substrate.[11,15] MshB activity was measured at 30 °C in assay mixtures containing 50 mM HEPES, 50 mM NaCl, 1 mM TCEP (pH 7.5), and 0−400 mM GlcNAc following the addition of enzyme (1−20 μM) as previously described.[14−16] Briefly, reaction aliquots (30 μL) were quenched (10 μL of 20% trichloroacetic acid), and the cleared supernatants (25 μL) were transferred to 96-well plates and diluted [75 μL of 1 M borate (pH 9)] prior to reaction with FSA [30 μL in CH_3CN (Invitrogen)].[14−16] Fluorescence measurements (excitation at 395 nm, emission at 485 nm) were taken using a SpectraMax M5^e platereader (Molecular Devices).

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The Michaelis−Menten equation was fit to the initial rates of product formation (<10%) to obtain parameters k_{cat}, K_M, and k_{cat}/K_M using the curve fitting program Kaleidagraph (Synergy Software).

RESULTS

Arg68 and Asp95 Are Critical for Molecular Recognition and Turnover of GlcNAc. Automated docking studies with MshB and GlcNAc suggest that the side chains of Arg68, Asp95, Tyr142, and His144 all make important hydrogen bonding interactions with the GlcNAc substrate (Figure 3),[12] which is consistent with the observed interactions between the GlcNAc core of GlcNAc-Ins and MshB from docking studies.[9,10,12] We previously confirmed the importance of the Tyr142 and His144 side chains using mutagenesis and kinetic studies.[16]

We have used a similar strategy of mutagenesis and enzyme kinetics to probe additional interactions predicted by automated docking studies with the substrate GlcNAc. Specifically, the side chains of Arg68 and Asp95 both share hydrogen bonds with the bound glycerol in the Act-MshB structure[10] and bound BOG in the BOG-MshB[9] structure and are predicted to share hydrogen bonds with the glucosamine hydroxyl groups on GlcNAc (Figure 3).[12] While Arg68 is located on helix α2 and appears to be relatively rigid, Asp95 is located on a mobile surface loop (residues 95−104). Removal of either side chain with the R68A or D95A mutant results in an inactive MshB enzyme that has no measurable activity (Table 1) with the GlcNAc substrate [≤100 mM GlcNAc (R68A) or ≤300 mM GlcNAc (D95A)]. These results indicate that the side chains of Arg68 and Asp95 are essential for the recognition and turnover of the GlcNAc substrate by MshB and provide the first biochemical confirmation of their importance.

Table 1. Steady-State Kinetic Parameters for Zn^{2+}-MshB Mutants with GlcNAc

MshB[a]	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	% of WT activity
WT[b]	38 ± 4	46 ± 2	20 ± 1	–
D15A[c]	52 ± 11	0.29 ± 0.02	0.09 ± 0.01	0.5
L19A	31 ± 2.2	43 ± 1.0	23 ± 1.2	115
E47A	17.5 ± 1.0	63 ± 0.9	60 ± 2.7	300
R68A	ND[d]	ND[d]	ND[d]	–[d]
D95A	ND[d]	ND[d]	ND[d]	–[d]
M98A	114 ± 11	20 ± 0.8	2.9 ± 0.2	15
D146A[c]	>400	>2	~0.08[c]	<0.4
D146N	63 ± 8	2.8 ± 0.10	0.74 ± 0.07	3.7
F216A	27 ± 1.4	36 ± 0.7	22 ± 0.8	110

[a]Apo-MshB was incubated with stoichiometric Zn^{2+} for 30 min prior to activity measurement. The substrate was GlcNAc (0−400 mM). [b]Data adapted from ref 14. [c]Data adapted from ref 16. [d]The activity of this mutant was not detectable.

The Hydrogen Bond at Position 146 Is Important for GlcNAc Recognition and Turnover. We have previously shown that Asp146 is important for recognition and turnover of GlcNAc by MshB, as the D146A mutant has ≤0.4% of WT MshB activity (Table 1).[16] However, this side chain is not positioned to make a direct binding interaction with substrate; rather, it is proposed that the Asp146 side chain may play a role in stabilizing a loop containing the Asp95 and Met98 side chains through hydrogen bonding interactions with Ser96, and possibly His13 (Figure 5A).[10] To test this hypothesis, we determined the steady-state parameters of the D146N mutant. The overall size of the Asn side chain is comparable to that of the natural Asp side chain, and it retains the ability to serve as a hydrogen bond donor and/or acceptor. It is expected that if Asp146 makes an important hydrogen bonding interaction in WT MshB, then the activity of D146N is greater than that of D146A. However, if the Asp side chain makes a critical ionic interaction in WT MshB, then the activity of D146N approximates that of D146A. Results for the D146N mutant (Table 1 and Figure 4) reveal that the values of k_{cat} and k_{cat}/K_M

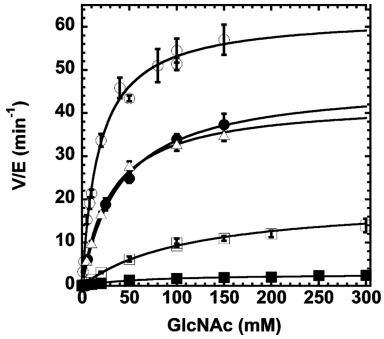

Figure 4. Catalytic activity of MshB variants. Steady-state turnover of GlcNAc catalyzed by Zn^{2+}-MshB variants: (●) WT MshB, (△) L19A, (○) E47A, (□) M98A, and (■) D146N. The initial rates for deacetylation of GlcNAc (0.1−400 mM) were measured at 30 °C in 50 mM HEPES, 50 mM NaCl, and 1 mM TCEP (pH 7.5), as described in Materials and Methods using MshB variants reconstituted with stoichiometric Zn^{2+}. The steady-state parameters (Table 1) were obtained by fitting the Michaelis−Menten equation to these data.

for the D146N mutant are significantly decreased (16- and 27-fold, respectively) compared to those of WT MshB, while the value of K_M is modestly increased (<2-fold change). However, the value of k_{cat}/K_M for the D146N mutant is ~10-fold higher than the k_{cat}/K_M value for the D146A mutant, and the value of K_M is significantly lower in the D146N mutant (63 mM) than in the D146A mutant (>400 mM). This enhanced activity of the D146N mutant versus that of the D146A mutant suggests that the D146N mutant is likely participating in a hydrogen bond interaction during recognition and turnover of GlcNAc by MshB.

Met98 Is Important for GlcNAc Recognition. Results from structure overlays and automated docking studies indicate that there are three glycine rich surface loops on MshB that are important for ligand recognition and catalysis, loop 1 (residues 95−104), loop 2 (residues 138−143), and loop 3 (residues 211−217),[8−10,12] as well as a hydrophobic cavity adjacent to the active site that may be an important determinant of MshB substrate specificity.[12,22] Loop 2 contains Tyr142, which we have previously shown to be a dynamic and catalytically important side chain (Table 1).[16] Herein, we examine the contributions of additional side chains located in the two remaining loops (i.e., Met98 and Phe216) as well as the hydrophobic cavity (i.e., Leu19 and Phe216) upon recognition of GlcNAc by MshB.

Surface loop 1 (residues 95−104) contains the side chains of Asp95 (probed above) and Met98, which is located at the entrance to the active site (Figure 6) and is proposed to regulate access to the active site, as well as provide a favorable hydrophobic interaction with the inositol moiety on GlcNAc-

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Ins to facilitate substrate binding.[12] While Met98 cannot make a similar hydrophobic interaction with the smaller GlcNAc substrate, in one of the MshB-GlcNAc docking conformations the S atom on Met98 is 3.0 Å from the 1-OH of GlcNAc (Figure 5A), suggesting it could accept a hydrogen bond from GlcNAc. To test this hypothesis, we examined the steady-state kinetics of the M98A mutant. Mutation to Met98 to Ala results in a 3-fold increase in the value of K_M^{GlcNAc} and a 7-fold decrease in the value of k_{cat}/K_M^{GlcNAc} (Figure 4 and Table 1), suggesting that the side chain of Met98 does play a role in GlcNAc recognition and turnover by MshB.

Surface loop 3 (residues 211−217) is proposed to form the outside wall of the hydrophobic cavity that binds the bimane groups on the larger amidase substrates (e.g., CySmB-GlcN-Ins, fCySmB-GlcN-Ins, and MSmB) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)-containing inhibitors.[12,22] Because the side chains of Tyr142 (loop 2) and Phe216 (loop 3) are located in the proximity of one another in crystal structures of some of the MshB monomers (Figure 5B), it is possible that a $\pi-\pi$ interaction between Tyr142 and Phe216 could be coupled to movement of Tyr142. To test this hypothesis, we examined the steady-state kinetics of the F216A mutant. Mutation of Phe216 to Ala does not significantly alter the kinetic parameters of MshB with the GlcNAc substrate (Table 1), suggesting movement of the catalytically important Tyr142 is not coupled to a $\pi-\pi$ interaction between this side chain and Phe216.

The side chain of Leu19 is located at the entrance of the hydrophobic cavity that is adjacent to the active site. The docking result for GlcNAc with MshB suggests that the acetyl group of GlcNAc may be too small to make direct contact with the Leu19 side chain (Figure 3, 3.6 Å). We decided to probe a potential interaction biochemically using the L19A mutant. While Ala is also a hydrophobic side chain, it is smaller in size and would increase the distance between the acetyl moiety on GlcNAc and the side chain at this position. Results for the steady-state kinetics (Figure 4 and Table 1) of the L19A mutant are consistent with the proposal that the acetyl group on GlcNAc is too small to interact with the hydrophobic cavity and influence recognition by MshB.

Mutation of Glu47 to Ala Enhances MshB Activity. Results from docking studies suggest that the side chain of Glu47 is located at the entrance of the active site (Figure 6) and that it stabilizes substrate binding via hydrogen bonding interaction(s) with the 3′-OH and 4′-OH on the inositol ring of GlcNAc-Ins.[10,12] Although Glu47 is not predicted to have any direct binding interactions with the smaller GlcNAc substrate,[12] we examined the effect of the E47A mutation on MshB activity with GlcNAc because this side chain is located at the entrance to the active site and could play a role in regulating access to the active site. Results of steady-state kinetic experiments (Figure 4 and Table 1) indicate that the E47A mutation decreases the value of K_M^{GlcNAc} (2-fold) and increases the value of k_{cat}/K_M^{GlcNAc} (3-fold), suggesting that loss of Glu47 enhances MshB activity with the GlcNAc substrate.

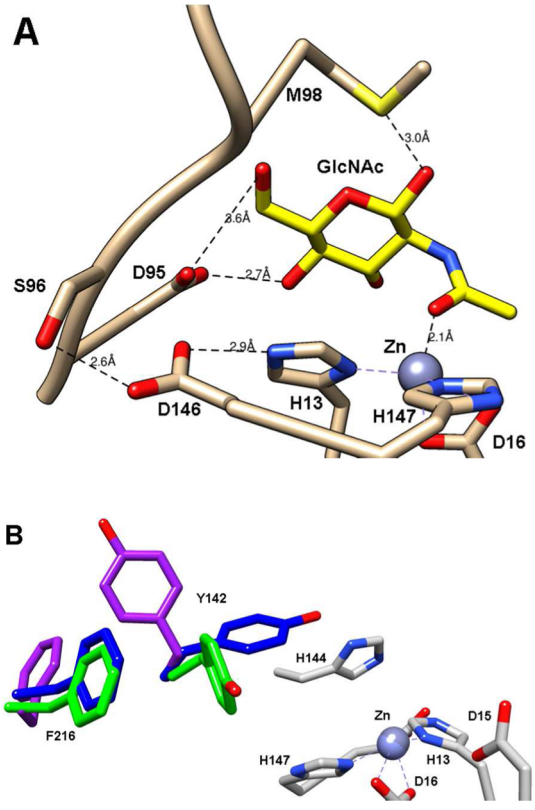

Figure 5. Close-up of predicted specific side chain interactions in the MshB-GlcNAc complex. (A) Asp146. Hydrogen bonding interactions between Asp146 and Ser96 (and possibly His13) may help to position and stabilize the mobile loop containing Asp95 and Met98, which make hydrogen bonding interactions with the bound GlcNAc substrate. (B) Phe216. An overlay of three MshB monomers (PDB entries 1Q74 and 1Q7T) where the side chains of Tyr142 and Phe216 are located in the proximity (~4−6 Å) of one another (each monomer is colored green, blue, or purple). Tyr142 and Phe216 are both located on mobile loops. Movement of the loops allows the Tyr142 and Phe216 side chains to move within 4 Å of one another, suggesting a possible $\pi-\pi$ interaction between the side chains.

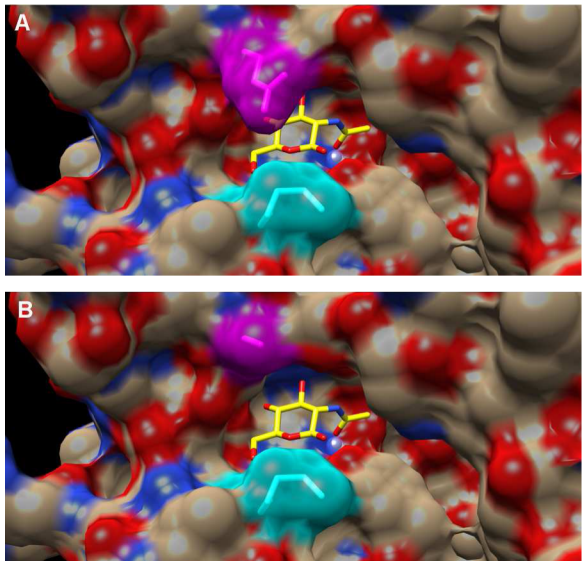

Figure 6. Surface area map illustrating MshB active site accessibility in the MshB-GlcNAc complex. (A) WT MshB. The substrate GlcNAc (yellow) is coordinated to the active site zinc ion (gray). The side chains of Glu47 (magenta) and Met98 (cyan) are located at the entrance to the MshB active site. (B) E47A mutant. This is a rendering of the viewpoint in panel A where the Glu47 side chain has been truncated to Ala.

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DISCUSSION

GlcNAc Binding Is Stabilized via Extensive Hydrogen Bonding Interactions. GlcNAc is proposed to bind to the MshB active site via extensive hydrogen bonding interactions between GlcNAc and the side chains of Arg68, Asp95, Tyr142, and His144 (Figure 3).[12] We previously confirmed the importance of the Tyr142 and His144 side chains in GlcNAc recognition and turnover using site-directed mutagenesis and enzyme kinetic experiments.[16] Importantly, we have now also confirmed the importance of the Arg68 and Asp95 side chains for GlcNAc recognition and turnover using similar site-directed mutagenesis and enzyme kinetic experiments (Figure 4 and Table 1). Removal of either side chain with the D95A and R68A mutants results in a complete loss of MshB activity with the GlcNAc substrate, confirming their importance for molecular recognition and turnover by MshB. One likely explanation for these findings is that these side chains make important binding interactions with substrate, such as the predicted hydrogen bonding interactions between these side chains and the GlcNAc substrate.

Effect of Asp146 on GlcNAc Recognition Likely Mediated through Other Side Chains. Results of these experiments have shown the ability of the D146N mutant to restore some of the MshB activity that was lost with the D146A mutant (Table 1), suggesting that the ability of the side chain at position 146 to participate in a hydrogen bonding interaction is an important factor for GlcNAc recognition. The decreased activity of D146N compared to that of WT MshB is likely explained by the fact that Asp is a better hydrogen bond acceptor than Asn. Because Asp146 is not positioned to make direct contact with GlcNAc (Figure 3), the importance of this side chain in ligand recognition is likely mediated through other side chain(s). The Asp146 side chain is within hydrogen bonding distance of two side chains, His13 and Ser96.[10,12] His13 is one of the ligands for the catalytic metal ion, while Ser96 is one of the side chains on mobile surface loop 2 (residues 95−104). Given that the D146A mutant does not appear to have grossly altered metal binding properties,[12] it is more likely that the observed effect is due to the hydrogen bonding interaction between Asp146 and Ser96 that helps to position and stabilize loop 2 containing the Asp95 and Met98 side chains that are critical for GlcNAc recognition (Figure 5A; see above).[10]

Met98 Might Share a Hydrogen Bond with GlcNAc. Results of mutagenesis experiments suggest a potential role for Met98 in the recognition of GlcNAc as the M98A mutant exhibits a 4-fold increase in its K_M^{GlcNAc} value (Figure 4 and Table 1), as well as ∼2- and 7-fold decreases in k_{cat} and k_{cat}/K_M, respectively. Met98 is located at the entrance to the active site and is proposed to regulate access to the active site, as well as provide a favorable hydrophobic packing interaction with the inositol moiety on the GlcNAc-Ins substrate.[10,12] The loss of activity observed with the smaller GlcNAc substrate is consistent with the proposal that Met98 serves as a hydrogen bond acceptor with the smaller GlcNAc substrate (Figures 3 and 5A) to facilitate GlcNAc binding and recognition. This role for Met98 would be precluded in the larger GlcNAc-Ins and amidase (e.g., CySmB-GlcN-Ins, fCySmB-GlcN-Ins, and MSmB) substrates where the 1-OH of GlcNAc is the site of attachment for the inositol moiety, although it could provide a favorable binding interaction with potential MshB inhibitors.

Glu47 May Contribute to Regulation of Active Site Access. Interestingly, the E47A mutation resulted in an ∼3-fold enhancement of MshB activity compared to that of the WT protein (Figure 4 and Table 1). While the Glu47 side chain is proposed to make important hydrogen bonding interactions with the inositol moiety on GlcNAc-Ins,[10,12] it does not appear to have any direct binding interactions with the smaller GlcNAc substrate (Figure 3). The finding that the activity with GlcNAc is enhanced with the E47A mutant could suggest that binding of GlcNAc to WT MshB is strained; however, none of the current structural or computational data suggest that this is true. A more likely explanation for these results is that the substitution of Glu47 with the smaller Ala at the entrance to the active site provides a wider opening (Figure 6), which allows for more rapid substrate binding and/or product release by MshB. It will be interesting to see the effect of the E47A mutation on GlcNAc-Ins recognition, because the loss of favorable binding affinity due to the loss of hydrogen bonding interactions may at least be partially offset by the wider opening to the active site.

Conclusions. Herein, we provide the first biochemical confirmation that the side chains of Arg68 and Asp95 are critical for recognition and turnover of the GlcNAc substrate, likely via hydrogen bonding interactions with the hydroxyl groups on the glucosamine moiety. Additionally, these studies suggest that the side chain of Met98 may have a direct favorable binding interaction (i.e., a hydrogen bond) with the smaller GlcNAc substrate. Finally, the side chain of Glu47 may help to modulate access to the enzyme active site as decreasing the size of the amino acid at this position enhances MshB activity. Other mutations examined (L19A and F216A) had more modest effects on MshB activity and, therefore, likely do not contribute significantly to the recognition and turnover of the GlcNAc substrate. Together, these results help to define the molecular determinants of GlcNAc recognition by MshB, an early step in trying to understand the molecular basis of the substrate specificity for this enzyme and an important step for the development of MshB inhibitors.

ASSOCIATED CONTENT

Supporting Information

Predicted protein stabilities and folding rates of MshB mutants (Table S1) and four additional references. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00068.

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Notes

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■ ABBREVIATIONS

Act-MshB, MshB complex with bound acetate and glycerol; BOG, β-octyl glucoside; CySmB-GlcN-Ins, bimane derivative of desacetylmycothiol; fCySmB-GlcN-Ins, formylated CySmB-GlcN-Ins; FSA, fluorescamine; GlcNAc, *N*-acetylglucosamine; 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; IMAC, immobilized metal ion affinity chromatography; MBP, maltose-binding protein; MSH, mycothiol; MSmB, acetylated bimane derivative of MSH; MshB, *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase; TCEP, tris(2-carboxyethyl)phosphine; TB, tuberculosis; TEV, tobacco etch virus.

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