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Protein Expression and Purification 47 (2006) 542–550
Protein Expression & Purification
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Purification and characterization of *Mycobacterium tuberculosis* 1D-*myo*-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside deacetylase, MshB, a mycothiol biosynthetic enzyme

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Received 19 October 2005, and in revised form 28 February 2006
Available online 29 March 2006

Abstract

Mycothiol (MSH, AcCys-GlcN-Ins) is the major low molecular weight thiol in actinomycetes and is essential for growth of *Mycobacterium tuberculosis*. MshB, the GlcNAc-Ins deacetylase, is a key enzyme in MSH biosynthesis. MshB from *M. tuberculosis* was cloned, expressed, purified, and its properties characterized. Values of k_{cat} and K_m for MshB were determined for the biological substrate, GlcNAc-Ins, and several other good substrates. The substrate specificity of MshB was compared to that of *M. tuberculosis* mycothiol *S*-conjugate amidase (Mca), a homologous enzyme having weak GlcNAc-Ins deacetylase activity. Both enzymes are metalloamidases with overlapping amidase activity toward mycothiol *S*-conjugates (AcCySR-GlcN-Ins). The Ins residue and hydrophobic R groups enhance the activity with both MshB and Mca, but changes in the acyl group attached to GlcN have opposite effects on the two enzymes.

Keywords: *Mycobacterium tuberculosis*; Mycothiol biosynthesis; MshB; Mca; Substrate specificity; Metalloamidase

Mycothiol (MSH, AcCys-GlcN-Ins, Fig. 1)[1] is the major non-protein thiol present in most actinomycetes and has functions analogous to those played by glutathione in glutathione-producing prokaryotes and eukaryotes [1]. Four genes encoding enzymes involved in MSH biosynthesis have been identified in *Mycobacterium tuberculosis* H37Rv (Fig. 1): a glycosyltransferase, (MshA; Rv0486) [2], a deacetylase (MshB; Rv1170) [3], a ligase (MshC; Rv2130c) [4], and an acetyltransferase, mycothiol synthase (MshD; Rv0819) [5]. Inactivation of MshC blocks MSH biosynthesis in *Mycobacterium smegmatis* but does not prevent growth [6]. However, the *mshC* gene is essential for growth of *M. tuberculosis*, indicating that MSH is also essential [7]. Thus, MshC, as well as other enzymes of MSH biosynthesis, are potential targets for drugs against *M. tuberculosis*. *M. smegmatis* appears to be different from *M. tuberculosis* in that it does not require MSH for growth [2,6,8].

The deacetylase, MshB, was the first enzyme of mycothiol biosynthesis to be identified and is related to mycothiol *S*-conjugate amidase (Mca), an enzyme that cleaves various *S*-conjugates of mycothiol (MSR; AcCySR-GlcN-Ins) to produce the mercapturic acid AcCySR and GlcN-Ins [9]. In *M. tuberculosis* MshB is not essential for synthesis of MSH or for growth, apparently because Mca has a low GlcNAc-Ins deacetylase activity that allows for slow MSH biosynthesis in the absence of MshB [10]. The crystal

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[1] *Abbreviations used:* AcCySR, a mercapturic acid; GlcNAc-Ins, 1-D-*myo*-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; Ins, *myo*-inositol; mBBr, monobromobimane; Mca, mycothiol *S*-conjugate amidase; MSH, mycothiol; MshA, first enzyme of MSH biosynthesis; MshB, *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase; MshC, ATP-dependent L-cysteine:1D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside ligase; MshD, mycothiol synthase; MSSM, mycothiol disulfide; MSR (AcCySR-GlcN-Ins), *S*-conjugate of mycothiol; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RSmB, monobromobimane derivative of thiol RSH; SDS, sodium dodecyl sulfate.

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doi:10.1016/j.pep.2006.03.003

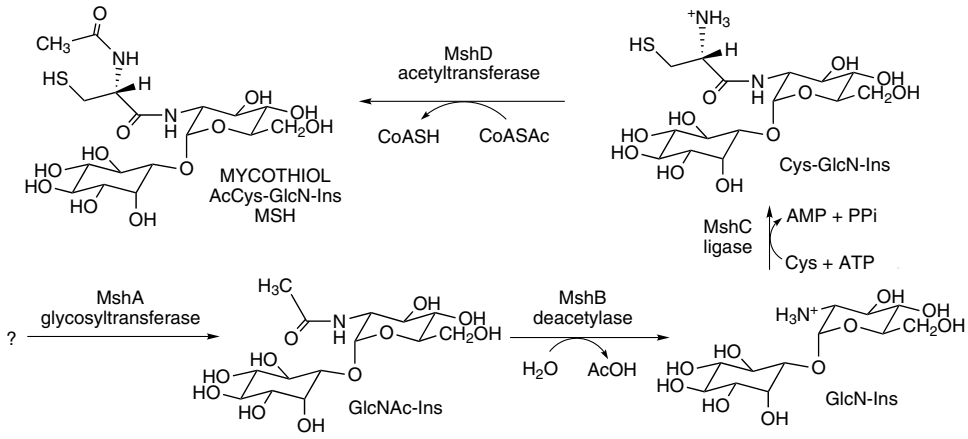

Fig. 1. Pathway for mycothiol biosynthesis.

structure of MshB has recently been published [11,12]. A zinc was identified in the active site and the N-terminal sequence containing the active site was shown to provide a good homology model for the 3D structure of Mca [11]. *M. tuberculosis* Mca has been cloned and expressed, and the purified enzyme shown to contain Zn and to have a broad spectrum of substrate activity [13].

Recently, considerable attention has been focused on identifying inhibitors of Mca and MshB as possible leads for drugs in the treatment of tuberculosis [14–20] and it is therefore important to have access to purified MshB for testing of inhibitors. The only published preparation for MshB was in connection with crystallographic studies [21] which produced protein that apparently lacked metal and was not reported to be active. In the present study, we describe the preparation of active *M. tuberculosis* MshB, document its requirement for divalent transition metals, characterize its kinetics and substrate specificity, compare its characteristics with those of Mca, and assess the common and differing substrate activities of the two enzymes.

Materials and methods

Materials

Mycothiol and mycothiol disulfide (MSSM) were prepared as described by Unson et al. [22]. Preparation of the bimane derivatives AcCySmB-GlcN, CySmB-GlcN-Ins, and formyl-CySmB-GlcN-Ins of the *N*-ethylmaleimide derivative of mycothiol (MS-NEM) and of the bromoacetophenone derivative of mycothiol (MS-acetophenone) were as described by Steffek et al. [13]. The iodoacetamide derivative (MS-acetamide) and the monobromobimane derivative (MSmB) of mycothiol have been described previously [9]. *N*-Acetylglucosamine was from Sigma and GlcNAc-Ins was prepared as described by Newton et al. [3]. Except as specified below, all other reagents were purchased from Fisher Scientific and were the highest grade available.

Cloning and expression of MshB

Rv1170 was cloned to contain a C-terminal His-6 tag to facilitate purification. Genomic DNA of *M. tuberculosis* H37Rv was prepared as previously described [23]. The open reading frame Rv1170 was amplified from this DNA with the primers 5′-TTCATATGGTGTCTGAGACGCCG-3′ and 5′-ATAAGCTTGTAGCCGGACGCGGTG-3′ containing *Nde*I and *Hind*III restriction sites, respectively. PCR was performed with *Taq* polymerase (Gibco BRL) using 1.5 mM $MgCl_2$ and 5% dimethylsulfoxide. The appropriate PCR product was ligated into the vector pCR2.1 of the TA cloning kit (Invitrogen) and transformed into *Escherichia coli* DH5α by standard chemical transformation. Plasmid DNA was digested with restriction endonucleases *Nde*I and *Hind*III (Fermentas) and the restriction enzyme-digested plasmids were isolated with a QIAquick gel extraction kit (Qiagen Ltd.). A corresponding digestion was applied to the plasmid pET-22b (Novagen) and the two products were ligated together with T4 DNA ligase to obtain the plasmid pYA1170c. This provides a recombinant protein without a pelB leader sequence, but with a C-terminal His-6 tag. This plasmid was transformed by the heat shock method to competent *E. coli* BL21(DE3) (Invitrogen). *E. coli* BL21(DE3) was also transformed with the blank cloning vector, pET16b, and inoculated in the same way as a control for SDS–PAGE.

Purification of MshB

Initial preparations of the pYA1170c protein were purified over a Ni^{2+}-affinity column and produced an active protein that contained no zinc and 0.82 mol of Ni^{2+} by inductively coupled plasma-atomic emission spectroscopy (ICP) analysis. Steffek et al. [13] showed that *M. tuberculosis* Mca, a close homolog of MshB, contained Zn^{2+} when expressed and purified from *M. smegmatis* without a His-6 tag. This indicated that Zn^{2+} was the native metal in MshB and all subsequent preparations of this protein were purified over a metal chelating resin charged with Zn^{2+}.

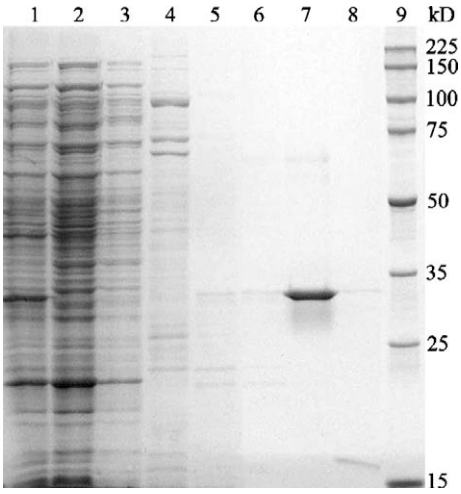

Fig. 2. SDS–PAGE analysis of the His-Bind Fractogel—Zn^{2+} purification of MshB with protein bands stained with Coomassie blue: lane 1, supernatant fraction; lane 2, unretained fraction; lane 3, buffer wash; lanes 4–5, 50 mM imidazole; lanes 6–8, 150 mM imidazole; and lane 9, molecular weight standards.

For isolation of MshB a 1 L culture was inoculated to 1% with a starter culture and incubated at 37 °C with shaking. Expression from the Rv1170 gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM when the A_{600} reached 0.5, and incubation was continued overnight at 22 °C before harvesting by centrifugation at 5000*g* for 20 min at 4 °C. The cells (5 g wet weight) were resuspended in 30 ml of lysis buffer: 50 mM NaCl, 50 mM Hepes (pH 7.5) containing 35 μM of each of the protease inhibitors *N*-*p*-tosyl-L-phenylalanine-chloromethyl ketone (Sigma) and *N*-*p*-tosyl-L-lysinechloromethyl ketone (Sigma). The cells were disrupted by sonication on ice, the crude homogenate was clarified by centrifugation (15,000*g*, 4 °C, 30 min), and further purification was conducted at room temperature. The soluble proteins were passed through a 0.5 × 5 cm column packed with His-Bind Fractogel M (Novagen) chelating resin in the Zn^{2+} form over a 1 h period. The column was washed with 40 ml of loading buffer to remove unbound proteins, and then step eluted with 25 ml each of loading buffer containing 50, 150, 250, and 500 mM imidazole. SDS–PAGE analysis showed that MshB eluted in a single 150 mM imidazole fraction with greater than 95% purity (Fig. 2). This fraction was concentrated using an Amicon Ultra 15 spin filter and the buffer exchanged by repeated washing (4 × 15 ml) with 50 mM Hepes (pH 7.5) containing 50 mM NaCl (assay buffer). After concentration, 10% glycerol was added to the purified deacetylase (3 mg) for a final volume of 3 ml and frozen at −70 °C.

Enzyme assays

The deacetylase activity was assayed with GlcNAc-Ins [3] as substrate and determination of the GlcN-Ins produced using a minor modification of the method of Anderberg et al. [24]. The standard assay protocol was conducted by equilibrating 80 μl of assay buffer containing 0.1 mM GlcNAc-Ins at 37 °C. Reaction was initiated by addition of 1.5 μg of enzyme in 1 μl of assay buffer. Samples (20 μl) were taken at intervals and the reaction was terminated by addition of an equal volume of acetonitrile containing 10 mM NEM (Sigma) and 2 mM 1,10-phenanthroline (Kodak) followed by incubation at 60 °C for 10 min. After cooling on ice and centrifugation for 3 min at 14,000*g* at room temperature, an aliquot of the supernatant was derivatized with AccQ-fluor (Waters) and assayed by HPLC method 5 as previously described [24].

The amidase activity was assayed by quantitation of the monobromobimane (mBBr, Molecular Probes) derivative of *N*-acetylcysteine (AcCySmB) produced during hydrolysis of MSmB. A representative assay (80 μl) contained 0.1 mM MSmB, 50 mM Hepes chloride, pH 7.5, and 50 mM NaCl prewarmed to 37 °C; 1.5 μg of enzyme was added to initiate reaction. Samples (20 μl) were removed at intervals and the reaction quenched by mixing with 60 μl of 40 mM methanesulfonic acid on ice. After centrifugation for 3 min at 14,000*g* in a microcentrifuge at room temperature, the supernatant was analyzed by HPLC with fluorescence detection as detailed elsewhere [5].

Initial deacetylase and amidase rates were determined by linear extrapolation of the rates calculated from analyses at 3, 6, and 10 min to zero reaction time.

Molecular mass determination

The active molecular mass of MshB was determined by gel filtration on a 1.4 × 90 cm column of Sephadex G100 equilibrated in 50 mM Hepes, pH 7.5, and calibrated with cytochrome C, trypsin inhibitor, ovalbumin, bovine serum albumin, and phosphorylase B, all supplied by Sigma.

Protein determination and sequencing

Protein concentration was routinely determined by the method of Bradford (Bio-Rad Laboratories). The protein concentrations of stocks for metal ion determination were analyzed for amino acid content following hydrolysis under vacuum for 20, 40, and 60 min at 160 °C as previously described [25]. The amino terminal protein sequence of recombinant MshB was determined on an Applied Biosystems Model 494 Procise gas phase protein sequencer at the UCSD Department of Biology Protein Sequencing Facility.

Metal ion content

The metal ion content of recombinant MshB was determined by ICP-AES analysis at the Environmental Laboratory of the San Diego Gas and Electric Company. Analyses included results for Al, Sb, As, Ba, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Mo, Ni, K, Se, Si, Na, Sr, Tl, V, and Zn.

Inactivation by phenanthrolines

Stock solutions (5 and 50 mM) of 1,10-phenanthroline and of 1,7-phenanthroline (Aldrich) were prepared in

dimethylsulfoxide (Sigma). MshB (6 μg) in 80 μl of assay buffer containing 1.6 μl of phenanthroline stock solution was incubated at 37 °C for 10 min. The deacetylase reaction was initiated by addition of 0.8 μl of 10 mM GlcNAc-Ins. A control reaction contained 1.6 μl of dimethylsulfoxide with no phenanthroline. Aliquots (20 μl) were taken at intervals, mixed with a equal volume of warmed 10 mM NEM in acetonitrile, and incubated at 60 °C for 10 min to terminate the reaction. The samples were cooled on ice and clarified by centrifugation for 5 min at 14,000*g* and 7.5 μl aliquots were derivatized with AccQ-Fluor (Waters) for analysis by HPLC as described previously [24]. Controls conducted with dimethylsulfoxide alone established that it had no effect on the assay.

Determination of K_m and k_{cat} values

The kinetic constants for catalysis by MshB of the deacetylation of GlcNAc-Ins and of the amidase reactions of MSmB and CySmB-GlcN-Ins were determined at 37 °C in 50 mM Hepes buffer, pH 7.5, containing 50 mM NaCl. The deacetylase activity was assayed at nine concentrations of GlcNAc-Ins (10–2000 μM). For the amidase reactions, ten concentrations of MSmB, and eight concentrations of CySmB-GlcN-Ins were employed, both in the range 10–10,000 μM.

Results

Cloning, purification, and properties of MshB

The ORF encoding *M. tuberculosis* MshB (Rv1170) was originally identified from the activity of the protein cloned into pET16b to give plasmid pYA1170b which was expressed in *E. coli* [3]. However, when attempts were made to obtain pure MshB from the transformed *E. coli*, the yields were poor, apparently owing to degradation of the active protein during the purification to a smaller inactive component detected previously in crude extracts [3]. In order to overcome this difficulty, Rv1170 was recloned to contain a C-terminal His-6 tag to permit its rapid isolation by affinity chromatography on either a Ni or Zn metal chelate resin. For purification on the Zn-affinity resin, fractions were collected during elution of the affinity column with imidazole and the purity of the eluted protein was assayed by SDS–PAGE (Fig. 2). The initial 50 mM imidazole fractions were contaminated with *E. coli* proteins, but a single 150 mM imidazole fraction was found to be >95% pure (Fig. 2). It was used to provide a stock of MshB and the purification is summarized in Table 1. A subunit M_r of 32,000 was obtained from the SDS–PAGE analysis (Fig. 2) and there was no evidence of the prominent $M_r = 26{,}000$ proteolytic product observed with the original Rv1170 construct.

The amino terminal sequence for MshB was determined to be MVSETPRKLFVHAHPDDESLSNGATIAHY with a minor contaminant of a second identical sequence having the Met residue deleted. This accords with the sequence of Rv1170 (GenBank accession number NP 215686; [26]) except for the insertion of a valine at residue 2 as prescribed by the 5′ primer sequence employed. This insertion was included in anticipation of the N-terminal Met being deleted by *E. coli*. The recombinant protein amino acid composition (data not shown) was determined to be within 10% of that expected when account was taken of the modified C-terminal sequence, ASGYKLAAALEH HHHHH. LC–MS analysis yielded two co-eluting peaks with masses of 33424.6 and 33293.0 Da, which corresponded within the experimental uncertainty to the parent protein (calculated 33423.2 Da) and the protein with N-terminal Met deleted (calculated 33292.0), respectively. Based upon the protein content from amino acid composition of the deacetylase, a value of $\varepsilon_{280} = 45{,}000 \pm 6000\ M^{-1}$ ($n = 5$) was determined for MshB. The predicted extinction coefficient was 44,500 M^{-1} as calculated from the amino acid sequence by the method of Gill and von Hippel [27].

Table 1
Purification of recombinant *M. tuberculosis* MshB

Steps	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Recovery (%)
Crude extract[a]	65	1060	16	100
Zn^{+2} chelate				
Affinity column	3.0	570	190	54

[a] From 1 L of bacterial culture.

The native molecular mass of purified MshB was determined by gel filtration on Sephadex G100. When the protein was loaded and eluted in 50 mM Hepes, pH 7.5, containing 0.1 mM $ZnCl_2$ it eluted as a single peak at a volume corresponding to an M_r of 79,000. When this peak was collected and reapplied to the same column equilibrated in 25 mM Hepes, pH 7.5, with 0.1 mM $ZnCl_2$, the activity eluted in two peaks, one at an $M_r \sim 158{,}000$ (beyond the linear separation range of the column) and a second at $M_r \sim 82{,}000$. Thus, the enzyme appears to behave as a dimer at normal ionic strength but may associate to a tetramer at low ionic strength.

MshB contains a divalent transition metal ion essential for activity

Metal analysis by ICP-AES of MshB isolated on the Ni-affinity column showed that it contained 0.36 equiv of Ca and 0.82 equiv of Ni, but <0.08 equiv of Zn per subunit. This enzyme was readily inactivated by 1,10-phenanthroline, a potent metal chelator (Fig. 3). This chelator produced 82% inhibition at 0.1 mM and 99.8% inhibition at 1.0 mM. No inhibition was observed with 0.1 mM 1,7-phenanthroline, a non-chelating isomer of 1,10-phenanthroline, and at 1 mM this isomer produced slight enhancement of activity (Fig. 3). Thus, the inhibition by 1,10-phenanthroline is not a general property associated with the phenanthroline heteroaromatic nucleus and indicates that the metal is required for activity of MshB. MshB isolated on

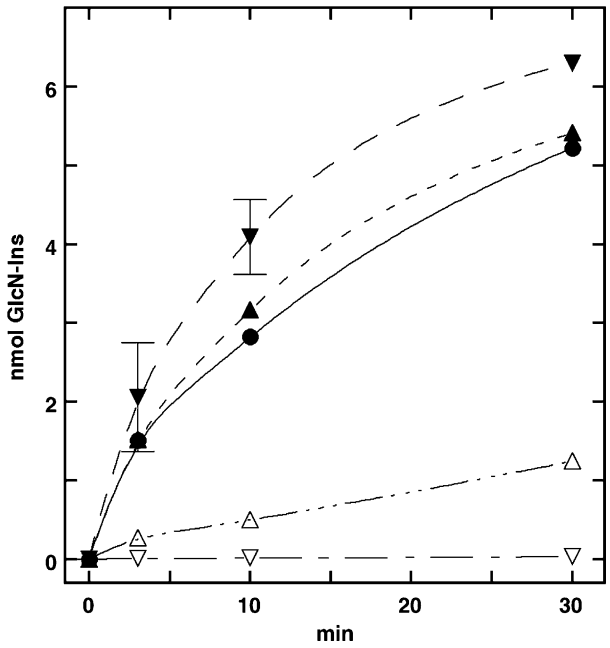

Fig. 3. Deacetylase activity of MshB (production of GlcN-Ins from 0.1 mM GlcNAc-Ins) in buffer alone (●), in buffer with 0.1 mM (△) or 1.0 mM (▽) 1,10-phenanthroline (metal-chelating) or in buffer with 0.1 mM (▲) or 1.0 mM (▼) 1,7-phenanthroline (non-metal-chelating). Range for duplicate determinations indicated by error bar when greater than the size of the symbol.

the Zn-affinity column contained <0.1 equiv of Ca and 2.3 ± 0.1 ($n = 2$) equivalents of Zn, but <0.08 equiv of Ni per subunit. When the Zn enzyme was treated with 1,7-phenanthroline the results were the same as for the Ni enzyme (Fig. 3). However, 0.1 mM 1,10-phenanthroline produced no significant inhibition and 10% activity remained after treatment with 1 mM 1,10-phenanthroline. This indicates that inhibition of the Zn enzyme by 1,10-phenanthroline is slower or less complete than inhibition of the Ni enzyme. MshB activity lost by incubation of the Ni enzyme with 1,10-phenanthroline could be restored following removal of 1,10-phenanthroline by incubation with 100 μM Zn^{2+}, Ni^{2+}, Mn^{2+}, or Co^{2+}, the latter promoting the highest activity, but Ca^{2+} and Mg^{2+} produced no restoration of activity (data not shown). Thus, a variety of transition metals can activate MshB but Zn^{2+} is the metal found in Mca, a homolog of MshB, when isolated without the use of a metal-affinity column [13] and therefore Zn^{2+} is assumed to be the metal present in native MshB.

Table 2
Activities (nmol/min/mg) of *M. tuberculosis* MshB and Mca with various substrates at 0.1 mM

Substrate	MshB[a]	Mca[b]
Deacetylase		
GlcNAc-Ins[c]	220 ± 40	0.055 ± 0.002
GlcNAc[d]	3 ± 2	
MSH (-Ac)[e]	<0.04	
MSmB (-Ac)[f]	< 0.01	
Amidase		
MSH (-AcCys)[c]	0.6 ± 0.2	9.5 ± 1.5
MSSM[c]	<0.2	64 ± 23
MSmB[c], AcCySmB-GlcN-Ins	58 ± 1	9900 ± 2000
AcCySmB-GlcN[d]	<0.2	0.22 ± 0.02
CySmB-GlcN-Ins[c]	1080 ± 10	18 ± 2
Formyl-CySmB-GlcN-Ins[g]	92 ± 9	660 ± 60
MS-acetamide[c]	0.69 ± 0.07	
MS-NEM[c]	6 ± 1	2000 ± 400
MS-acetophenone[c]	146 ± 6	8600 ± 600

[a] Assayed in 50 mM Hepes, 50 mM NaCl, pH 7.4, at 37 °C with 7.5–75 ng/μl MshB.
[b] Calculated from data in Ref. [13] for assay in 50 mM Hepes, 50 mM NaCl, pH 7.5, at 37 °C with 0.28–28 ng/μl of Mca.
[c] Reaction assayed for GlcN-Ins.
[d] Reaction assayed for GlcN.
[e] Reaction assayed for Cys-GlcN-Ins.
[f] Reaction assayed for CySmB-GlcN-Ins.
[g] Reaction assayed for formyl-CySmB.

The substrate specificity of MshB overlaps that of Mca

A variety of compounds were examined as substrates for deacetylase and amidase activities of MshB and the results are presented in Table 2. The structures of the substrates tested, the sites of hydrolysis, and the relative rates for MshB are shown in Fig. 4. Also included in Table 2 are the corresponding activities for Mca calculated from published data [13]. MshB had greatest deacetylase activity with GlcNAc-Ins and was about two orders of magnitude less active with GlcNAc. The enzyme was unable to remove the acetyl residue from the AcCys group of MSH or MSmB, and it exhibited barely detectable amidase activity with MSH or MSSM. However, it did have significant amidase activity with MSmB and even greater activity with CySmB-GlcN-Ins, formyl-CySmB-GlcN-Ins, and MS-acetophenone. A lower, but still significant, amidase activity was found with MS-NEM.

MshB kinetics

Three key substrates identified in the survey, GlcNAc-Ins, MSmB, and CySmB-GlcN-Ins, were studied in greater detail. Kinetic constants for these substrates (Table 3) were obtained from least squares analysis of Eadie–Hofstee plots of the data. Surprisingly, CySmB-GlcN-Ins is a better substrate than GlcNAc-Ins as measured by both K_m and k_{cat} values.

Discussion

MshB was first cloned into pET16b (pYA1170b) to yield a protein with an N-terminal His-6 tag and was found to be active in crude extracts of *E. coli* [3]. As mentioned above, this construct lost activity during purification with the appearance of a prominent 26 kD proteolytic product. The current construct (pYA1170c) contains a C-terminal His-6 tag and was found to be stable and easily purified (Fig. 2). A reason for the great difference in stability of these two constructs became apparent with availability of the crystal structure of MshB [11]. The N-terminal of MshB contains a

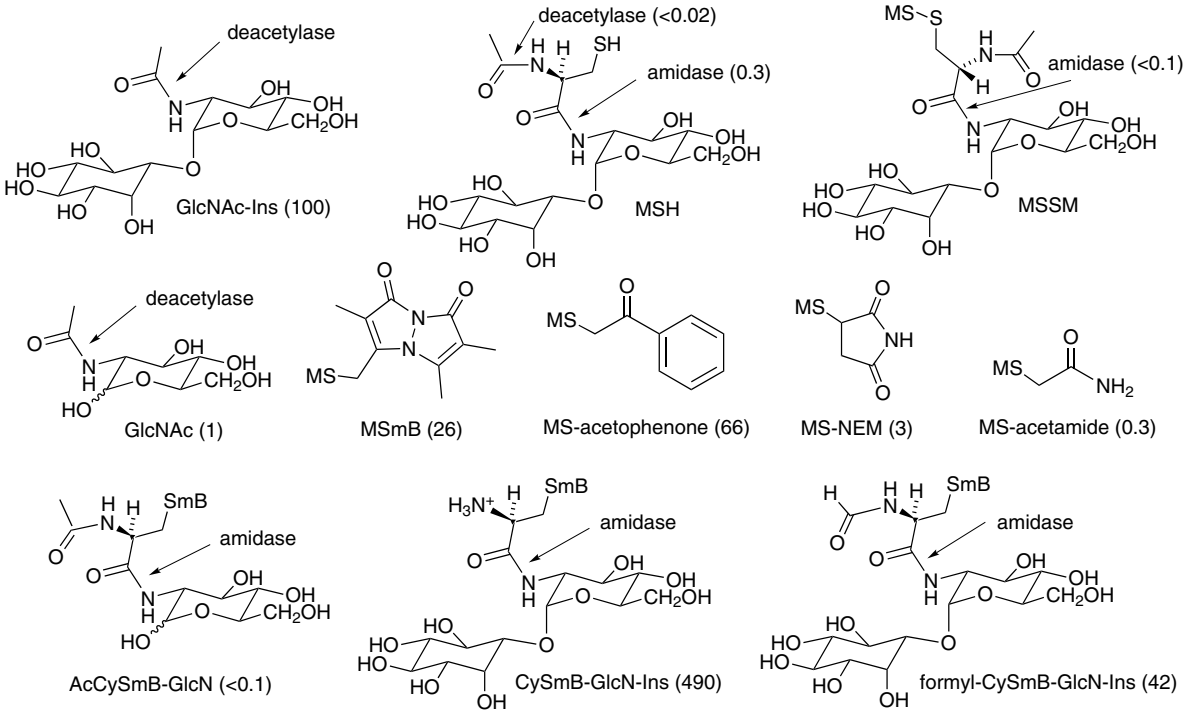

Fig. 4. Substrates examined with MshB. Bonds potentially cleaved in deacetylase and amidase reactions with each substrate are indicated by arrows. Relative activity is indicated in parenthesis as percentage of GlcNAc-Ins activity.

highly conserved 13HPDD16 sequence (*M. tuberculosis* numbering), which forms the metal binding active site in conjunction with H144 and H147. Residues H13, D15, and D16 are directly involved in metal binding or catalysis. The first construct with its N-terminal His-6 tag placed a high affinity divalent metal binding site near the active site zinc ion. This may explain why this activity was unstable and the protein prone to proteolytic cleavage.

The C-terminal His-6 tagged MshB was originally isolated on a Ni-affinity column and was found to contain 0.82 equiv of Ni per subunit. When the homologous enzyme, Mca, was cloned from *M. tuberculosis* without a His-6 tag and isolated from *M. smegmatis* without use of an affinity column, analysis showed that it contained 1.4 equiv of Zn per subunit [13]. Since this indicated that Zn was the native metal, subsequent purification of MshB was conducted on a Zn-affinity column and produced enzyme having 2.3 equiv of Zn per subunit, the additional Zn possibly associated with the His-6 tag. X-ray crystallographic studies of enzyme purified on a Ni-affinity resin showed that a metal is associated with the active site, but the crystals were generated from enzyme prepared on a nickel-affinity resin and would be expected from the present results to have a Ni^{2+} at the active site. The metal ion analysis reported as unpublished data from the current authors [11] was for MshB prepared on a Zn-affinity resin so the assignment of Zn^{2+} as the active site metal ion in crystals used for X-ray crystallography rests on the X-ray fluorescence studies and active site electron density, both of which were consistent with the presence of zinc. It is possible that the native Zn^{2+} is retained during purification on a Ni-affinity resin under some conditions. Since the Ni and Zn enzymes have nearly identical enzyme activity, it is unlikely that the protein structures are significantly different at the active site. The results indicate that various transition metal ions can activate MshB, including Zn^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+}, a feature common to many metallopeptidases [28].

Table 3
Kinetic constants for MshB

Parameter	Substrate		
	GlcNAc-Ins	MSmB	CySmB-GlcN-Ins
K_m (μM)	340 ± 80	500 ± 50	134 ± 10
V_{max} (nmol/min/mg)	960 ± 150	330 ± 20	2820 ± 150
k_{cat} (s^{-1})	0.49 ± 0.04	0.23 ± 0.01	3.6 ± 0.4
k_{cat}/K_m ($M^{-1}\,s^{-1}$)	1440 ± 360	460 ± 50	27,000 ± 3600

The results from the phenanthroline inhibition studies clearly indicate that the divalent metal ion is essential for the activity of MshB (Fig. 3). Analogous results were found with the homologous Mca [13]. The metal binding ligands identified by X-ray crystallography of MshB [11] are fully conserved in the Mca sequences of diverse gram positive bacteria [12]. A number of studies have identified inhibitors of Mca and many of these include oxime and other potential metal binding moieties [18–20].

MshB was found to be active as a dimer by gel filtration, and at lower ionic strength there was evidence of a higher oligomer. In this respect it behaves differently from the related mycothiol *S*-conjugate amidase (Mca) isolated from *M. smegmatis* which eluted at low ionic strength primarily as a monomer but with a small amount of activity eluting at a position indicating an oligomer larger than a dimer [9].

A goal of the present study was to compare the substrate specificity of MshB with that of the Mca cloned from *M. tuberculosis* [13]. The latter enzyme has a 170-fold higher specific activity than MshB when assayed with 0.1 mM MSmB under the same conditions (Table 2). Based upon results for the *M. smegmatis* enzymes ([9]; G. Newton, unpublished results) both proteins are present at similar levels, ~0.04% of the total cell protein. Thus, the major amidase activity toward mycothiol *S*-conjugates in the cell is provided by Mca. Conversely, the major GlcNAc-Ins deacetylase activity is provided by MshB. With 0.1 mM GlcNAc-Ins as substrate MshB has 4700-fold greater activity than Mca (Table 2) and MshB is therefore the dominant producer of GlcN-Ins for mycothiol biosynthesis.

It was of interest to examine the activities of these enzymes with other substrates (Table 2 and Fig. 4). Removal of Ins from MSmB to generate AcCySmB-GlcN decreases the activity with both enzymes, a factor of >290 for MshB and a factor of 45,000 for Mca. In addition, GlcNAc-Ins was 87-fold more reactive than GlcNAc with MshB. Thus, the Ins residue is important for binding to both enzymes. However, the structural specificity for the Ins moiety may be low since the replacement of the inositol residue in MSmB by a cyclohexane reduces amidase activity with Mca by only ~50% [16]. MS-NEM is a better amidase substrate than MSH by a factor of 10 for MshB and a factor of 200 for Mca. MSmB is 100-fold more reactive than MSH with MshB and almost 1000-fold more reactive with Mca. For both enzymes attachment of hydrophobic groups to the sulfur enhances the amidase activity.

A key region of difference between Mca and MshB involves the interaction with the acyl group attached to GlcN-Ins. This is apparent in the comparison of GlcNAc-Ins with MSH, where the acetyl group becomes replaced by AcCys. This change results in a 430-fold decrease in activity with MshB but a 180-fold increase in activity with Mca (Table 2). Since both enzymes presumably have the same catalytic mechanism for cleavage at this acyl group, these reactivity differences suggest that there must be important structural differences for substrate binding between MshB and Mca very near the catalytic site. The active site of Mca involves residues 10–23, which are fully conserved in diverse species of actinomycetes [13]. This region is also identical with the corresponding region (residues 11–24) of MshB, with the exception of residues 19–22 which are totally different (LSNG vs. SKGA). This may be associated with the differential binding of the residues in the region of the acyl group, but a crystal structure with a bound substrate, or closely related inhibitor, is needed to test this hypothesis.

Of the substrates tested, the one exhibiting the best compromise of activity with both MshB and Mca was MS-acetophenone (Table 2). For MshB it had 66% of the activity measured with GlcNAc-Ins and for Mca it showed 87% of the activity found with MSmB. In order to disrupt MSH biosynthesis at the deacetylase step in *M. tuberculosis* it is probably necessary to inhibit both MshB and Mca due to the overlapping deacetylase activity [10]. MS-acetophenone is a good substrate for MshB and Mca, indicating that a single inhibitor may be able to inactivate both enzymes. A bromotyrosine inhibitor has been obtained from a natural products screen that inhibits both Mca and MshB [14,18].

A rather surprising result was the finding that CySmB-GlcN-Ins is a better substrate for MshB than its natural substrate GlcNAc-Ins. The greater reactivity of CySmB-GlcN-Ins is reflected in both the K_m and the k_{cat} values (Table 2). It seems unlikely that this has any physiological significance. The concentration of Cys-GlcN-Ins in cells is almost unmeasurable [24] so it seems highly unlikely that its *S*-conjugate could be formed in significant amount by alkylation of its thiol group in competition with the analogous reaction by MSH, present at a 1000-fold higher concentration. In contrast, CySmB-GlcN-Ins is one of the poorest substrates tested for Mca. It is possible that the substitution of Ser19 in MshB by Lys in Mca disfavors binding of this positively charged substrate.

The structure–activity studies also produced results that had immediate practical consequences. The finding that MS-NEM is a significant substrate for MshB called into question our previous method for estimating the GlcNAc-Ins content of cells [3]. This depended upon utilizing MshB to deacetylate GlcNAc-Ins in an extract in which MSH had been blocked with NEM. The GlcN-Ins was then determined by fluorescent labeling and HPLC analysis as a measure of the GlcNAc-Ins content. Since MshB can act upon both GlcNAc-Ins and MS-NEM to release GlcN-Ins, this method can potentially overestimate the GlcNAc-Ins content. The results of Table 2 show that MSH and MSSM are substantially poorer substrates for MshB so the protocol was modified to eliminate the NEM from extract preparation and this modification eliminates the artifacts produced by the NEM protocol [10].

There appear to be some important differences between *M. tuberculosis* and *M. smegmatis* with regard to the consequences of inactivating *mshB*. The *mshB* mutant in *M. tuberculosis* produces MSH at ~20% of the wild type level in early exponential growth with the GlcNAc-Ins content at 40 times the normal level and 15 times the MSH level. The MSH level increases ~25-fold with growth and eventually exceeds the normal MSH level in stationary phase [10]. The results are consistent with the production of MSH by the low GlcNAc-Ins deacetylase activity of Mca ([10]; Table 2). By contrast, the level of MSH found in an *M. smegmatis mshB* mutant was ~10% of the wild type level during early exponential growth and it increased less than 2-fold in stationary phase; GlcNAc-Ins levels were not reported [29]. Moreover, overexpression of the *M. tuberculosis mca* gene in the *M. smegmatis mshB* mutant had no effect upon the MSH content. This is difficult to understand unless a substantially lower GlcNAc-Ins content in the *M. smegmatis* mutant, coupled with its more rapid growth, make the contribution of the *M. tuberculosis* Mca undetectable in the *mca* supplemented strain.

In conclusion, a convenient preparation for the MshB deacetylase of *M. tuberculosis* is now available. MshB shares many properties with its homolog, the mycothiol *S*-conjugate amidase Mca, including an active site divalent transition metal, amidase activity with a wide range of substrates, and GlcNAc-Ins deacetylase activity. The activity of both enzymes is reduced by elimination of the Ins residue but is enhanced by hydrophobic groups attached to the sulfur of mycothiol. Surprisingly, CySmB-GlcN-Ins proved to be a better substrate for MshB than its natural substrate whereas it was a rather poor substrate for Mca.

Acknowledgments

We thank Teresa Koledin for technical assistance, Micah Steffek for assistance with the gel filtration studies, and Stephen Smith for assistance with the amino acid analyses. This work was supported by grants to R.C.F. from the National Institute of Allergy and Infectious Diseases (AI49174) and the Fogarty International Center (TW00976) and to Y.A. from the British Columbia Lung Association, and by contracts supporting R.C.F. and Y.A. from eXegenics Incorporated. R.C.F and Y.A. were consultants for eXegenics Incorporated during a portion of these studies. We thank Thomas Black of the Environmental Analysis Laboratory, San Diego Gas and Electric for providing the ICP-AES analyses and Jason Maynes of the University of Alberta for thoughtful discussions.

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