

A Novel Mycothiol-Dependent Detoxification Pathway in Mycobacteria Involving Mycothiol S-Conjugate Amidase[†]

Gerald L. Newton,[‡] Yossef Av-Gay,[§] and Robert C. Fahey^{*‡}

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093 and Department of Medicine, University of British Columbia, Vancouver, British Columbia V5Z 3J5

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ABSTRACT: Mycothiol, 1-D-*myo*-inosityl-2-(*N*-acetylcysteinyl)amido-2-deoxy- α -D-glucopyranoside (MSH), is composed of *N*-acetylcysteine (AcCys) amide linked to 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (GlcN-Ins) and is the major thiol produced by most actinomycetes. When *Mycobacterium smegmatis* was treated with the alkylating agent monobromobimane (mBBBr), the cellular mycothiol was converted to its bimane derivative (MSmB). The latter was rapidly cleaved to produce GlcN-Ins and the bimane derivative of *N*-acetylcysteine (AcCySmB), a mercapturic acid that was rapidly exported from the cells into the medium. The other product of cleavage, GlcN-Ins, was retained in the cell and utilized in the resynthesis of mycothiol. The mycothiol S-conjugate amidase (amidase) responsible for cleaving MSmB was purified to homogeneity from *M. smegmatis*. A value of $K_m = 95 \pm 8 \mu\text{M}$ and a value of $k_{\text{cat}} = 8 \text{ s}^{-1}$ was determined for the amidase with MSmB as substrate. Activity with 100 μM mycothiol or with the monobromobimane derivative of 1-D-*myo*-inosityl-2-(L-cysteinyl)amido-2-deoxy- α -D-glucopyranoside (CySmB-GlcN-Ins) or of 2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-(α,β)-D-glucopyranoside (AcCySmB-GlcN) was at least 10^3 lower than with 100 μM MSmB, demonstrating that the amidase is highly specific for S-conjugates of mycothiol. Conjugates of mycothiol with the antibiotic cerulenin, *N*-ethylmaleimide, 3-(*N*-maleimidopropionyl)-biocytin, and 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin also exhibited significant activity. The sequence of the amino-terminal 20 residues was determined, and an open reading frame (Rv1082) coding for 288 residues having an identical predicted amino-terminal amino acid sequence was identified in the *Mycobacterium tuberculosis* genome. The Rv1082 gene (*mca*) from *M. tuberculosis* was cloned and expressed in *Escherichia coli*, and the expressed protein was shown to have substrate specificity similar to the amidase from *M. smegmatis*. These results indicate that mycothiol and mycothiol S-conjugate amidase play an important role in the detoxification of alkylating agents and antibiotics.

Actinomycetes do not make glutathione, an antioxidant considered to play a key role in protection of cells against oxygen toxicity and electrophilic xenobiotics but do produce millimolar levels of mycothiol (MSH, AcCys-GlcN-Ins),¹ an unusual conjugate of *N*-acetylcysteine (AcCys) with 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside (GlcN-Ins) (1). Mycothiol autoxidizes more slowly than glutathione (2), and mutants of *Mycobacterium smegmatis* defective in the biosynthesis of mycothiol have increased sensitivity to hydrogen peroxide relative to the parent strain (3). This observation suggests that mycothiol may play a key role in the protection of actinomycetes against oxygen toxicity. The biochemistry of mycothiol appears to have evolved completely independently of that of glutathione, so elucidation of the details of the metabolism of mycothiol and comparison with the established roles for the metabolism of glutathione

is expected to provide insights into the roles played by thiols in detoxification.

It has already been established that the metabolism of mycothiol parallels that of glutathione metabolism in two enzymatic processes. First, formaldehyde is detoxified in glutathione-producing organisms by NAD/glutathione-dependent formaldehyde dehydrogenase (4, 5). An analogous process involving NAD/mycothiol-dependent formaldehyde dehydrogenase has been identified in the actinomycete

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^{*} To whom correspondence should be addressed. Telephone: 858-534-2163. Fax: 858-534-4864. E-mail: rcfahy@ucsd.edu.

[‡] The University of California, San Diego.

[§] The University of British Columbia.

¹ Abbreviations: AccQ-fluor, 6-aminoquinolyl-*N*-hydroxysuccinimide carbamate; AcCys, *N*-acetylcysteine; AcCys-GlcN, 2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-(α,β)-D-glucopyranoside; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; Cys-GlcN-Ins, 1-D-*myo*-inosityl-2-(L-cysteinyl)amido-2-deoxy- α -D-glucopyranoside; GlcN-Ins, 1-D-*myo*-inosityl-2-amino-2-deoxy- α -D-glucopyranoside; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; mBBBr, monobromobimane; MPB, 3-(*N*-maleimidopropionyl)biocytin; MSA, iodoacetamide S-conjugate of mycothiol; MSC, cerulenin S-conjugate of mycothiol; MSH, mycothiol, 1-D-*myo*-inosityl-2-(*N*-acetylcysteinyl)amido-2-deoxy- α -D-glucopyranoside; MSME, NEM S-conjugate of mycothiol; MSMC, CPM S-conjugate of mycothiol; MSMPB, MPB S-conjugate of mycothiol; MSsB, sBBBr S-conjugate of mycothiol; MSqB, qBBBr S-conjugate of mycothiol; NEM, *N*-ethylmaleimide; qBBBr, monobromotrimethylammoniumbimane; RDW, residual dry weight; RSmB, bimane derivative of corresponding thiol RSH; sBBBr, *p*-sulfo benzoyloxybromobimane; Tris, tris(hydroxymethyl)aminomethane.

Amycolatopsis methanolica (6). This enzyme has been sequenced (7), and the sequence has a homologue (Rv2259) in the genome of *Mycobacterium tuberculosis* with 80% amino acid sequence identity. Homologues with similar identity were found in the databases for *Mycobacterium leprae* (Sanger) and *Mycobacterium avium* (TIGR). This enzyme therefore appears to have broad distribution among actinomycetes. Second, a mycothiol homologue of glutathione reductase was recently cloned from *M. tuberculosis* and expressed in *M. smegmatis* (8, 9). The reductase is reasonably specific for the disulfide of mycothiol but is also active with the disulfide of AcCys-GlcN, the desmyo-inositol derivative of mycothiol (9).

In the present studies, we describe a pathway of detoxification dependent on mycothiol in which an alkylating agent is converted to a *S*-conjugate of mycothiol, the latter is cleaved to release a mercapturic acid, and the mercapturic acid is excreted from the cell. This process has similarities to the mercapturic acid pathway for glutathione-dependent detoxification in higher eukaryotes (10) but involves fewer steps. The amidase responsible for cleavage of the *S*-conjugate of mycothiol has been purified from *M. smegmatis* and characterized, and the *M. tuberculosis* homologue (Rv1082) has been cloned and expressed in *Escherichia coli*.

MATERIALS AND METHODS

Enzymatic Assays. The enzymatic activity was routinely assayed by quantitation of the bimeane derivative of *N*-acetylcysteine (AcCySmB) produced from the bimeane derivative of mycothiol (MSmB), prepared from purified mycothiol (2). Separation of the various modified thiols was performed by high-pressure liquid chromatography (HPLC). A sample (2–10 μ L) of extract was mixed with 40 μ L of 30 μ M MSmB in 3 mM 2-mercaptoethanol, 25 mM HEPES chloride, pH 7.5, and reacted 10–30 min at 30 °C before quenching the reaction with 50 μ L of 40 mM methanesulfonic acid on ice. The mixture was centrifuged for 3 min at 14000g in a microcentrifuge at room temperature, and the supernatant was analyzed by HPLC without dilution. A shortened version (45 min) of HPLC method 1 of Fahey and Newton (11) was used for separation of MSmB and AcCySmB. The bimeane derivative of mycothiol eluted at 23.5 min, and AcCySmB eluted at 27 min.

A preparation of the mycothiol *S*-conjugate amidase (30–50% saturated ammonium sulfate fraction chromatographed on Sephadex G-75) was used to study the stoichiometry of the reaction. Reaction was initiated by mixing a sample (9 μ L, 7 μ g of total protein) of the preparation of the enzyme with 0.9 mL of 50 mM sodium phosphate, pH 7.5, containing 100 μ M MSmB. For determination of bimeane derivatives of thiols, a sample (70 μ L) of reaction mixture was removed, mixed with 4 μ L of 5 M methanesulfonic acid, and analyzed by HPLC without dilution. For analysis of GlcN-Ins, a sample (2–8 μ L) of the reaction mixture was mixed with enough 1M HEPES chloride, pH 8, to bring the volume to 10 μ L and then with 5 μ L of acetonitrile and 5 μ L of 10 mM AccQ-Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, Waters). The mixtures were incubated for 1 min at room temperature followed by 10 min at 60 °C, diluted with 60 μ L of water, and quantified by HPLC as previously described (12).

The specificity of the amidase for substrate was assessed by measuring the production of GlcN-Ins in most cases. A sample (5 μ L) of 1 mM substrate was mixed with 40 μ L of 3 mM 2-mercaptoethanol, 25 mM HEPES chloride, pH 7.5. The reaction was initiated with 5 μ L of purified amidase (50-fold diluted stock, 4.4 μ g mL⁻¹). Triplicate samples were quenched at 0, 10, and 30 min by mixing each sample with 50 μ L of acetonitrile containing 5 mM NEM and incubating at 60 °C for 10 min. After the samples were cooled on ice, they were clarified by centrifugation for 15 min at 14000g. A sample (15 μ L) of the supernatant was modified with AccQ-Fluor for amine analysis in a total reaction volume of 125 μ L as previously described (12).

Preparation of GlcN-Ins. Salt-free, stereochemically pure GlcN-Ins was prepared from MSmB by enzymatic hydrolysis using partially purified amidase. A sample of MSmB purified by HPLC (4.8 μ moles) was incubated in 2 mL of water without buffer at 23 °C with 15 μ g of partially purified amidase. The reaction was monitored hourly for its content of MSmB and AcCySmB, and the pH was adjusted to 7.5 with 1 M NaOH as necessary. Additional aliquots of enzyme were added as necessary to achieve complete hydrolysis of the MSmB over a maximum interval of 12 h, after which the reaction mixture was acidified to a pH less than 3 with trifluoroacetic acid (Fluka). A 1-mL SepPak, C18 cartridge (Waters) was prepared by sequentially washing with 5 mL of methanol, 5 mL of 50% methanol, 0.1% trifluoroacetic acid in water, and 20 mL 0.1% trifluoroacetic acid in water. The acidified reaction mixture was applied, and the column was eluted with 0.1% trifluoroacetic acid. Fractions (1 mL) were collected and analyzed for their content of GlcN-Ins. The fractions containing GlcN-Ins were pooled, lyophilized, and resuspended in a small volume of water. Complete hydrolysis of MSmB is important because AcCys-mB and protein, but not MSmB, are retained on the SepPak C18 cartridge under these conditions.

Analysis of *M. smegmatis* Treated with mBBR in Culture. A logarithmic phase culture (1 L) of *M. smegmatis* mc²155 (OD₆₀₀ = 1.2) in 7H9 Middlebrook medium was cooled on ice to 3 °C. The iced culture was incubated with mBBR (0.5 mM from a 180 mM stock solution in acetonitrile) for 20 min; excess 2-mercaptoethanol (1.0 mM) was added, and the incubation continued on ice for an additional 10 min. The cells were pelleted by centrifugation and washed twice with 200 mL of sterile, ice-cold 7H9 Middlebrook medium to remove excess bimeane derivative of 2-mercaptoethanol. The cells were resuspended in 50 mL of sterile ice-cold medium, and the experiment was initiated (*t* = 0) by dilution into 950 mL of prewarmed (37 °C) 7H9 Middlebrook medium. The cell suspension was shaken in an incubator (225 rpm, 37 °C) and a “*t* = 0” sample (100 mL) was removed from the culture within 1 min. This sample was mixed with an equal weight of ice and stored on ice until the second sample was taken similarly at 5 min, after which time both were pelleted at 5000g and 4 °C. Additional samples were obtained in this manner at times up to 4 h. An aliquot (0.5 mL) of supernatant was mixed with 0.5 mL of acetonitrile and incubated at 60 °C for 10 min. After centrifugation, the supernatant was assayed by HPLC for bimeane-labeled thiols in the medium. The pellet (always iced) was separated into three roughly equal parts in 1.5-mL microcentrifuge tubes and extracted for 10 min in three ways

as follows. The first sample was extracted using 1 mL of 60 °C acetonitrile–water for determination of cellular thiol-bimane derivatives. The second sample was extracted using 1 mL of 50% acetonitrile–water (60 °C) containing 2 mM mBBR and 20 mM Tris, pH 8.0, for determination of the sum of each cellular thiol and thiol-bimane derivative. The third sample was extracted using 1 mL of 50% acetonitrile–water (60 °C) containing 5 mM *N*-ethylmaleimide (NEM) and 10 mM HEPES chloride, pH 7.5. All tubes were centrifuged at 14000g in a microcentrifuge, and the supernatants were removed for analysis. For determination of GlcN-Ins, 0.1 mL was removed from supernatant 3, and 15 μ L was assayed in a total assay volume of 125 μ L as previously described (12). The remaining 0.9 mL of supernatant 3 was derivatized with 2 mM mBBR and 20 mM Tris, pH 8.0, to serve as the NEM control for the analysis of sample 2. All assay pellets were dried in a vacuum oven and weighed to obtain the residual dry weight (RDW) for calculation of results.

Purification of Mycothiol S-Conjugate Amidase. *M. smegmatis* cells were cultured as above to late log phase, collected by centrifugation at 5000g, and frozen at –70 °C until used. Thawed cell paste (100 g) was mixed with 500 mL of 3 mM 2-mercaptoethanol, 25 mM HEPES chloride, pH 7.5 (assay buffer), without protease inhibitors and disrupted by sonication on ice. The extract was centrifuged for 30 min at 15000g at 4 °C, and the supernatant was mixed with saturated ammonium sulfate to 20% saturation and incubated for 1 h on ice. After centrifugation at 15000g and 4 °C for 30 min, the pellet was discarded. The supernatant was adjusted to 50% saturated ammonium sulfate, incubated on ice overnight, and centrifuged for 30 min at 10000g and 4 °C. The pellet was resolubilized in 60 mL of cold assay buffer and dialyzed against assay buffer overnight at 4 °C.

The dialyzed sample was applied to a 1.4 \times 27 cm column of Toyopearl DEAE 650C (TosoHaas), and the column was washed with \sim 3 column volumes of assay buffer at 4 °C. The column was developed with a linear gradient in assay buffer from 0 to 0.4 M NaCl, and the amidase activity eluted at \sim 0.2 M NaCl. The active fractions were pooled, and saturated ammonium sulfate was added to 20% saturation. After 1 h on ice, the solution was clarified by centrifugation for 30 min at 10000g, and the pellet was discarded. The supernatant was applied to a 1.4 \times 27 cm column of Phenyl Sepharose 4B (Sigma) equilibrated with 20% saturated ammonium sulfate in assay buffer at 4 °C. The column was washed with 5 column volumes of 20% saturated ammonium sulfate followed by 5 column volumes of 10% saturated ammonium sulfate, both in assay buffer. The Phenyl Sepharose 4B column was eluted in assay buffer with a linear gradient from 10 to 0% saturated ammonium sulfate, and the amidase activity eluted at \sim 1–2% saturated ammonium sulfate. The active fractions were pooled and concentrated at 4 °C using a Biomax-50 (Millipore) ultrafilter.

The concentrated activity pool was applied to a Sephadex G-100 (Pharmacia) column (1.8 \times 88 cm) equilibrated with assay buffer. The majority of the activity eluted at an estimated M_r of 36 000. The most active peak fractions were pooled and concentrated on Centricon C-30 (Amicon) ultrafilters at 4 °C. Purified amidase was stored in assay buffer containing 20% glycerol at –70 °C for at least 12 months without significant loss of activity.

Characterization of Mycothiol S-Conjugate Amidase. The level of purification and subunit molecular weight was estimated on 12% SDS–polyacrylamide slab gel electrophoresis (13) calibrated with broad range standards (Bio-Rad). The native molecular weight was estimated based upon three preparative scale chromatographies on Sephadex G-100 used as the final step of purification and described above. The column was calibrated with dextran blue, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme, all from Sigma. The amino-terminal sequence of the purified amidase was determined on an Applied Biosystems Procise model 494 gas-phase protein sequencer by the UCSD Department of Biology Protein Sequencing Facility.

Preparation of Mycothiol S-Conjugates. Mycothiol S-conjugates were prepared by reaction of excess electrophile with mycothiol followed by removal or reaction of excess electrophile. Stock solutions (100 mM) of electrophile were prepared in acetonitrile (NEM, iodoacetamide; Sigma) or in dimethyl sulfoxide [7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM, Molecular Probes), 3-(*N*-maleimidopropionyl)biocytin (MPB, Sigma)]. Reaction with these electrophiles at 2 mM in 25 mM HEPES chloride, pH 7.5, was initiated by addition of mycothiol to 1 mM from a 32 mM stock solution in H₂O (pH \sim 3) and was allowed to proceed for 15 min in the dark. Excess reagent was removed by extracting 3 times with an equal volume of H₂O-saturated dichloromethane. A cerulenin (Sigma) stock solution (100 mM in acetonitrile) was diluted 5-fold in 25 mM HEPES chloride, pH 7.5, reacted for 15 min with 1 mM mycothiol, and extracted 5 times with H₂O-saturated dichloromethane. Prior to extraction, a 1 μ L aliquot was withdrawn for reaction with mBBR and analysis of residual mycothiol by HPLC. This showed that >99% of the mycothiol had reacted. Stock solutions (50 mM) of sBBR and qBBR were prepared in 50% aqueous dimethyl sulfoxide and diluted to 2 mM for reaction with 1 mM mycothiol in 25 mM HEPES chloride, pH 7.5, for 15 min. Since these charged electrophiles cannot be extracted with dichloromethane, they were reacted with excess (1.1 mM) 2-mercaptoethanol. In control studies, it was shown that 100 μ M levels of these 2-mercaptoethanol derivatives do not inhibit the amidase-promoted hydrolysis of MSmB, and it was assumed that their presence does not influence the reaction rate with other bimane derivatives of mycothiol.

Cysteine/GlcN-Ins Ligase Assays. The purified *M. smegmatis* amidase was assayed for ATP-dependent cysteine: GlcN-Ins ligase activity essentially as described by Anderberg et al. (12). As a positive control, a cell extract was prepared from *M. smegmatis* logarithmic phase cells disrupted by sonication on ice in 3 mM 2-mercaptoethanol in 50 mM HEPES chloride, pH 7.5. The cell debris was pelleted by centrifugation at 14000g for 3 min, and the supernatant was dialyzed against 3 mM 2-mercaptoethanol in 50 mM HEPES chloride, pH 7.5, overnight at 4 °C. The purified amidase or dialyzed *M. smegmatis* extract was incubated in 100 μ M cysteine, 50 μ M GlcN-Ins, 1 mM ATP, and 5 mM MgCl₂ in 50 mM HEPES chloride, pH 7.5, at 30 °C and assayed for the time-dependent formation of 1-D-myoinosityl-2-(L-cysteinyl)amido-2-deoxy- α -D-glucopyranoside (Cys-GlcN-Ins) by HPLC (12). The reaction was initiated with the addition of the purified amidase (0.044 μ g)

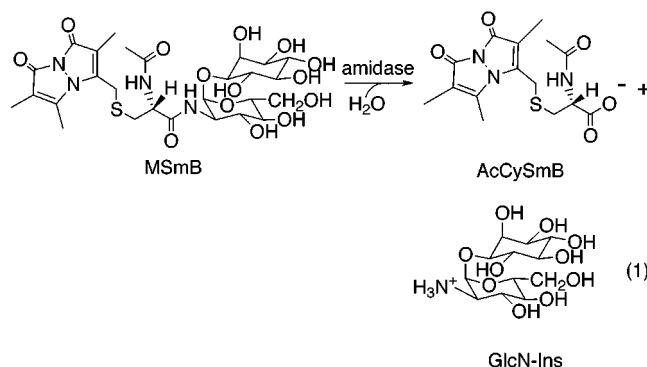
or cell extract (50 μ g of protein) and was sampled at 0 and 60 min.

Cloning and Expression of *M. tuberculosis* Rv1082. The *M. tuberculosis* gene Rv1082 was amplified from genomic DNA of *M. tuberculosis* H37Rv (NCTC 7416) using the following primers: 1, 5'-TAGCCATGGTGAGCGAACTGCG-GTTGATG-3' and 2, 5'-GGATCCCGATCCCGGCGAA-CAATTCGGT-3' as described elsewhere (14). *M. tuberculosis* Rv1082 was cloned into pET22b (Novagen) and used for transformation of *E. coli* BL21 (DE3) from Stratagene (14). Cells were cultured in Luria Bertani broth containing 100 μ g/mL ampicillin at 37 °C to OD₆₀₀ = 0.8 when isopropyl- β -D-thiogalactopyranoside was added to 0.4 mM, and the culture was shaken overnight at 25 °C. Cells were pelleted by centrifugation for 10 min at 5000g and 4 °C, sonicated in 5 vol of assay buffer on ice, and centrifuged for 5 min at 14000g at room temperature. About 85% of the amidase activity, using MSmB as substrate, was associated with the pellet fraction, which was resuspended and incubated with periodic vortexing for 1 h at 37 °C in 8 M urea containing 20 mM DTT. The suspension was centrifuged for 3 min at 14000g, and the supernatant was dialyzed against 100 vol of 25 mM HEPES chloride, pH 7.5, containing 1 mM glutathione disulfide and 2 mM glutathione for 15 h and then against 100 vol of 25 mM HEPES chloride, pH 7.5, containing 3 mM 2-mercaptoethanol for 4 h. After centrifugation, the supernatant contained soluble amidase activity and was assayed with 0.1 mM mycothiol and 0.1 mM MSmB as described above.

RESULTS

Alkylation of mycothiol with mBBBr produces the stable, fluorescent derivative MSmB that can be quantitated by HPLC (1, 15). However, when a pure sample of MSmB was exposed to a cell-free extract from *M. smegmatis*, the recovery of MSmB was poor and a substantial amount of AcCySmB was detected, indicating that MSmB had been cleaved by an enzyme present in the cell extract. The experiment was repeated using a partially purified cell extract, and samples of the incubation mixture were analyzed at intervals for the other potential product of MSmB cleavage, GlcN-Ins, as well as for MSmB and AcCySmB. On the basis of four determinations at >50% conversion, it was found that 1.0 equiv of MSmB (0.1 nmol) yielded 1.00 ± 0.02 equiv of AcCySmB and 0.80 ± 0.08 equiv of GlcN-Ins with the reaction proceeding to 97% conversion of MSmB in 60 min at 23 °C. This established the presence in the cell extract of mycothiol S-conjugate amidase that catalyzes the reaction shown in eq 1.

Since mBBBr is known to penetrate cells rapidly and to convert intracellular thiols to their bimane derivatives (15), we examined the fate of mycothiol in *M. smegmatis* cells treated with mBBBr to ascertain whether the reaction of eq 1 occurs in vivo. A logarithmic phase culture of *M. smegmatis* was cooled on ice to ~ 3 °C to reduce enzymatic reactions prior to reaction with 0.5 mM mBBBr. Excess reagent was reacted with 2-mercaptoethanol, and the cells were pelleted, washed, and resuspended in a small amount of ice-cold medium. The cells were diluted with prewarmed medium and replaced in the shaking incubator at 37 °C (time, $t = 0$). Samples were removed and analyzed for intracellular and



extracellular mycothiol related compounds over a 4-h interval (Figure 1).

At $t = 0$, no significant mycothiol or MSmB was found in cells or medium, but both contained significant levels of AcCySmB, indicating that the mycothiol had fully reacted with mBBBr and hydrolyzed to the corresponding AcCySmB derivative (Figure 1), much of this conversion presumably having occurred during the initial incubation on ice. At $t = 0$, 90 nmol/100 mL (3.5 μ mol/g RDW) of AcCySmB was present in the cells, and 160 nmol/100 mL was found in the medium. Within 5 min, the cellular AcCySmB level had fallen to 8 nmol/100 mL, and the medium level increased to 220 nmol/100 mL. Subsequent analyses found essentially all of the AcCySmB in the medium at a level of 225–230 nmol/100 mL, accounting for 80–90% of the original cellular mycothiol content. No MSmB appears to be exported from cells as <1 nmol/100 mL (<0.5%) was detected in the medium or the cell washes.

The cellular level of GlcN-Ins was comparable to that of AcCySmB at $t = 0$ (Figure 1) and represented a ~ 25 -fold increase above the normal level of ~ 0.1 μ mol of GlcN-Ins/g RDW (12). The GlcN-Ins level declined slowly over the 4-h incubation, while the mycothiol content increased from a nearly undetectable level at $t = 0$ to about half the normal cellular level after 4 h (Figure 1). These results indicate that GlcN-Ins produced by cleavage of MSmB is retained by the cell and is utilized in the resynthesis of mycothiol. No detectable low molecular weight bimane derivatives remained in the cells at 4 h. During the 4-h incubation, the A_{600} value initially decreased $\sim 15\%$ but then recovered its initial value of 1.2. Continued incubation at 37 °C resulted in a further increase to 1.6 at 8.5 h and a final $A_{600} \sim 2.6$ at 30 h. Thus, at least one cell doubling occurred after the treatment with mBBBr, and the cells entered stationary phase at a normal cell density.

Next we undertook purification of the enzyme responsible for cleavage of MSmB from *M. smegmatis* (Table 1). A 20–50% saturated ammonium sulfate fraction was chromatographed on a DEAE resin, then on a Phenyl Sepharose column, and finally on Sephadex G-100 (Figure 3). The three center fractions from the main peak of the G-100 chromatography had specific activities of ~ 3000 nmol/min-mg of protein with 30 μ M MSmB as substrate and were pooled to provide a pure amidase preparation. This sample produced a single band on SDS gel electrophoresis. Only the peak fractions of activity from the gel filtration step were selected, and this was the principle factor in reducing the overall yield to 11% (Table 1).

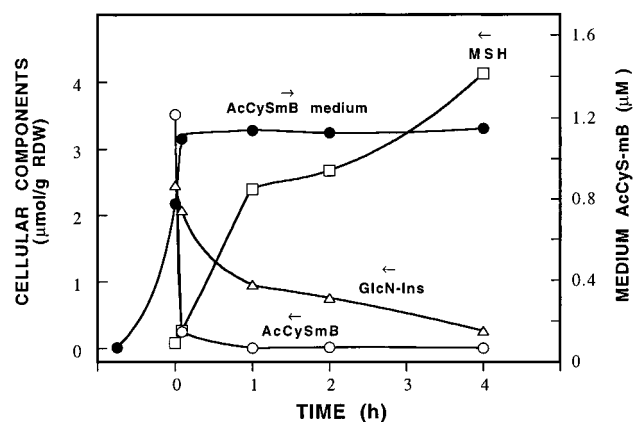


FIGURE 1: Detoxification of mBBR by exponentially growing cells of *M. smegmatis*. *M. smegmatis* treated with 0.5 mM mBBR at 3 °C, washed free of mBBR, and resuspended in fresh medium at 37 °C at time = 0; AcCySmB content of cells (○) and medium (●); cellular content of MSH (□) and GlcN-Ins (△).

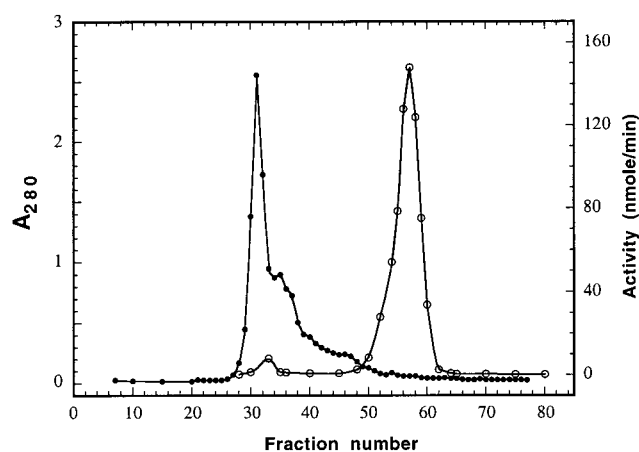


FIGURE 2: Final step in *M. smegmatis* amidase purification. Sephadex G-100 chromatography of highest specific activity fractions from Phenyl-Sepharose chromatography: A₂₈₀ (●); amidase activity (○).

A subunit M_r for the amidase of 36 000 was estimated based upon three or more determinations in each of three independent SDS–polyacrylamide slab gels. A native M_r of 36 000 was determined for the main peak of activity on three independent preparative Sephadex G-100 chromatographic separations. This indicates that the amidase is active as a monomer. A small and variable amount of activity eluted in the void volume (Figure 2), prior to bovine serum albumin ($M_r = 68\,000$). Thus, an aggregate larger than a dimer, or a larger protein with related catalytic activity, may be present. The activity from the preceding Phenyl Sepharose step was concentrated using a 50 000 cutoff filter, and no activity was found in the filtrate, suggesting that at high protein concentration the enzyme may be aggregated.

Table 1: Purification of *M. smegmatis* Mycothiol S-Conjugate Amidase

purification step	protein (mg)	total activity (units) ^a	specific activity (units/mg) ^a	yield (%)	purif factor
crude extract	8700	10.6	0.0012	100	1
20–50% ammonium sulfate pellet	3600	6.3	0.0018	59	1.5
DEAE 650C	2100	7.5	0.0036	71	3.0
Phenyl Sepharose	76	9.4	0.123	89	103
Sephadex G-100	0.35	1.16	3.3	11	2800

^a Assayed with 30 μM MSmB in assay buffer.

Table 2: Substrate Specificity of *M. smegmatis* Mycothiol S-Conjugate Amidase^a

amidase substrates	activity (nmole min ⁻¹ mg ⁻¹)	relative activity (%)
mycothiol conjugate with		
control ^b	13 ± 11	0.29 ± 0.25
mBBR (MSmB)	4480 ± 870	(100)
sBBR (MSsB)	740 ± 94	17 ± 2
qBBR (MSqB)	620 ± 44	14 ± 1
cerulenin (MSC)	370 ± 20	8.0 ± 0.5
iodoacetamide (MSA)	21 ± 2	0.47 ± 0.04
NEM (MSME)	107 ± 9	2.1 ± 0.2
CPM (MSMPC)	183 ± 50	3.8 ± 1.1
MPB (MSMPB)	246 ± 12	5.2 ± 0.3
other substrates		
mycothiol (MSH)	4 ± 1	0.1
mycothiol disulfide (MSSM)	25 ± 1	0.6
CySmB-GlcN-Ins	<4.5 ^c	<0.1
AcCySmB-GlcN	<0.8 ^d	<0.02

^a Measuring GlcN-Ins production from 0.1 mM substrate by 0.022 μg of purified amidase at 30 °C except as noted. ^b With 0.1 mM MSmB in the absence of amidase; calculated for 0.022 μg of enzyme. ^c Rate determined from CySmB formation. ^d Rate determined from AcCySmB formation.

The kinetics of the purified amidase were studied by assaying production of AcCySmB from MSmB. The pH dependence was examined in 50 mM sodium phosphate buffer. No significant dependence upon pH was detected from pH 7 to 9, but the activity declined sharply below pH 7 using 100 μM MSmB as substrate. At pH 7.5, activity was ~25% lower in 50 mM sodium phosphate than in 25 mM HEPES chloride, and the latter buffer was used for further studies. Values of $K_m = 95 \pm 8 \mu\text{M}$ and $k_{cat} = 8 \pm 1 \text{ s}^{-1}$ (site $M_r = 36\,000$) were determined from an Eadie–Hofstee plot based upon 16 determinations at MSmB concentrations from 1 μM to 3 mM.

The substrate specificity of the amidase reaction was examined using various mycothiol-related compounds (Table 2; Figures 3 and 4). A very low but measurable activity was found with mycothiol under these conditions. When myco-

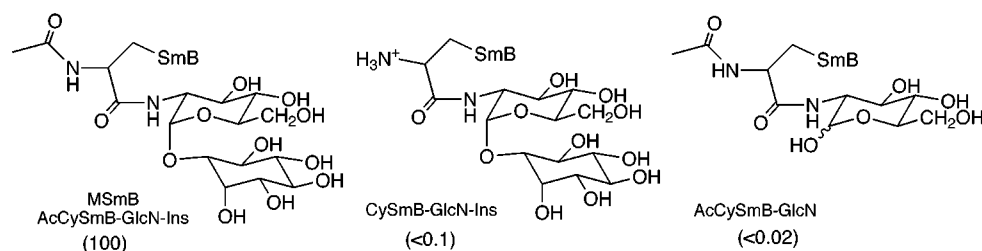


FIGURE 3: Substrate specificity of mycothiol S-conjugate amidase for changes in structure of the mycothiol moiety; MSmB activity defined as 100%.

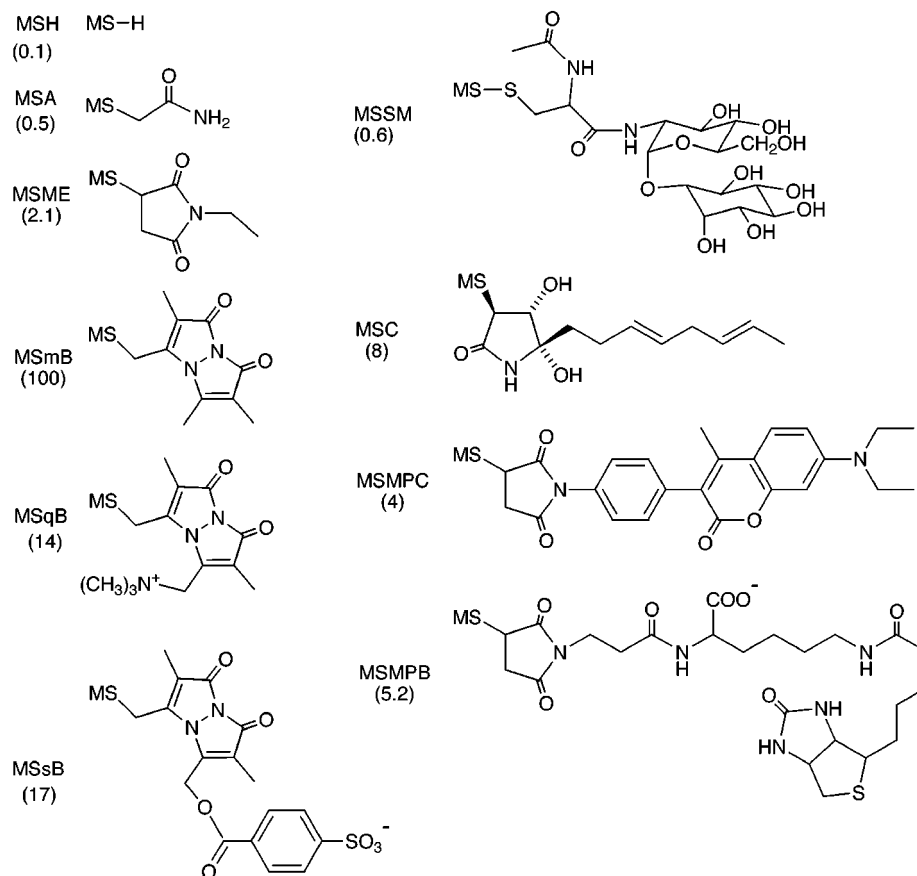


FIGURE 4: Substrate specificity of mycothiol *S*-conjugate amidase for changes in structure of the *S*-conjugate group; MSmB activity defined as 100%.

thiol was examined at 2 mM, a concentration comparable to the cellular level of mycothiol (12), under conditions otherwise the same as for Table 2, the rate of GlcN-Ins formation was $14 \pm 1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ or 0.3% of the rate with 0.1 mM MSmB. Mycothiol disulfide was a better substrate than mycothiol but still $<1\%$ as reactive as MSmB. Removal of the acetyl group from MSmB resulted in a 10^3 -fold reduction in rate, and removal of the inositol residue produced a $\sim 10^4$ -fold loss of activity (Figure 3). The amidase exhibited substantial activity with a wide range of *S*-conjugates other than MSmB, including the *S*-conjugate of the antibiotic cerulenin (16), which serves as an example of a naturally occurring substrate (Table 2).

We also tested whether mycothiol inhibits the cleavage of 100 μM MSmB by the amidase. The amidase activity was decreased by 30 ± 3 , 48 ± 3 , and $89 \pm 3\%$ ($n = 2$) at 1, 3, and 10 mM mycothiol, respectively. This suggests that cellular levels of mycothiol could produce significant inhibition of the monomeric amidase, the presumed form under these assay conditions.

Purified *M. smegmatis* mycothiol *S*-conjugate amidase was assayed for cysteine:GlcN-Ins ligase activity, a mycothiol biosynthesis enzyme (17). The reaction utilizes ATP to drive formation of an amide bond of the type hydrolyzed by the amidase. The purified amidase was incubated with ATP, cysteine, and GlcN-Ins, and the product, Cys-GlcN-Ins, was assayed by HPLC as the bimeane derivative (12). The purified amidase (0.044 μg) gave $<0.33 \text{ nmol min}^{-1} \text{ mg}^{-1}$ Cys-GlcN-Ins at a protein concentration in which the amidase reaction rate for 30 μM MSmB was $\sim 3000 \text{ nmol min}^{-1} \text{ mg}^{-1}$. As

a positive control, the ligase reaction was also assayed for a dialyzed crude extract from *M. smegmatis*, and 0.36 $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein Cys-GlcN-Ins was formed in accord with previous reports (3, 12). Thus, the amidase does not appear to be involved in mycothiol biosynthesis since it has no significant ability to catalyze ATP-dependent ligation of cysteine with GlcN-Ins. It therefore does not appear to be a bifunctional enzyme analogous to the glutathionylspermidine synthetase/amidase that catalyzes both the biosynthesis and the degradation of glutathionylspermidine in *E. coli* (18) and in *Crithidia fasciculata* (19). Attempts to detect mycothiol *S*-transferase activity in extracts of *M. smegmatis* using 1-chloro-2,4-dinitrobenzene with 1 mM MSH and monochlorobimane with 0.1 mM MSH did not produce significant activity (data not shown).

Sequencing of the amino-terminal portion of purified *M. smegmatis* amidase produced an amino-terminal sequence of (M)SELRLMAVHAHPDDESSKG. The first amino acid was not uniquely defined, and its assignment is uncertain. A BLAST search (Sanger Centre) of the *M. tuberculosis* H37Rv genome database (20) identified an open reading frame of unknown function, Rv1082, having an identical amino-terminal sequence and a M_r of 32 700. When *M. tuberculosis* Rv1082 gene was used to BLAST search the available databases, open reading frames with identical amino-terminal sequences and very high overall homology were found in genomes of *M. tuberculosis* CSU #93 (TIGR), *M. avium* (TIGR), *M. leprae* (Sanger Centre), and *M. bovis* (Sanger Centre).

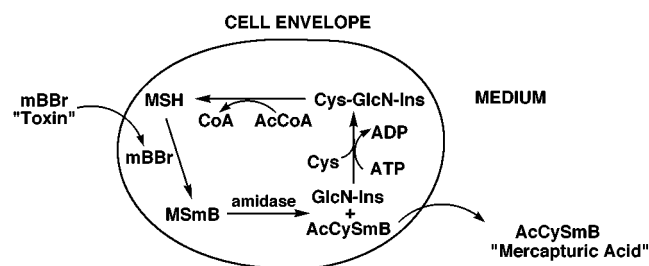


FIGURE 5: Schematic summary of MSH-dependent detoxification of mBBR by mycobacteria.

To verify the assignment of *M. tuberculosis* Rv1082 as a mycothiol conjugate amidase, we PCR-cloned the gene from *M. tuberculosis* H37Rv into *E. coli*. Extracts from uninduced cells showed $<0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$ amidase activity with $100 \mu\text{M}$ MSmB as substrate, whereas extracts of cells induced with isopropyl- β -D-thiogalactopyranoside produced $4.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$ amidase activity. *E. coli*, unlike *M. smegmatis*, does not have mycothiol metabolism (1), which accounts for the very low background activity of uninduced cell extract. When these extracts were assayed with 0.1 mM mycothiol as substrate $<0.002 \text{ nmol min}^{-1} \text{ mg}^{-1}$ amidase activity was observed. Thus, the recombinant *M. tuberculosis* amidase is more than 2000-fold more active with MSmB than with mycothiol itself, a substrate specificity very similar to that of *M. smegmatis* amidase (Table 2).

DISCUSSION

Exposure of *M. smegmatis* growing cultures to mBBR depletes cellular mycothiol by alkylation of the thiol group to form MSmB. No MSmB was found in the medium after *M. smegmatis* was treated with mBBR. Thus, MSmB is not exported from mycobacteria but is rapidly cleaved intracellularly by the amidase to produce GlcN-Ins plus AcCysmB. The AcCysmB is excreted from the cell, while GlcN-Ins is retained for resynthesis of MSH. This detoxification pathway is summarized in Figure 5.

AcCys S-conjugates are termed mercapturic acids, the final excreted product in the mercapturic acid pathway of glutathione-dependent detoxification in mammals (10). Although the mammalian glutathione-dependent and the mycobacterial mycothiol-dependent systems produce the same final product, the mammalian system is more complex. In mammals, intracellular conversion of alkylating agents to glutathione S-conjugates is catalyzed by glutathione S-transferases (21, 22) and occurs in various tissues. Glutathione S-conjugates are exported to the plasma and transported to other tissues, notably kidney and liver, where they are extracellularly degraded by γ -glutamyltranspeptidase to CySR-Gly, and the latter is cleaved by a dipeptidase to produce a cysteine S-conjugate, CySR (10). CySR is imported and acetylated by acetyl CoA to produce a mercapturic acid (AcCySR), which is ultimately excreted in urine and bile.

Although the final excreted product is the same in the two systems, the detailed biochemistry is different. Intracellular degradation of MSmB is advantageous for a single cell organism because the hydrophilic GlcN-Ins produced is retained by the cell and can be utilized for resynthesis of mycothiol. The more hydrophobic mercapturic acid, AcCysmB, is rapidly lost from cell. This loss may occur by passive diffusion or could be facilitated by a specific export

system. Since AcCys is a component of mycothiol, the mycothiol-dependent detoxification of electrophiles requires only a single enzyme, mycothiol S-conjugate amidase, to cleave the S-conjugate and produce the mercapturic acid excreted by the cell.

Other glutathione-producing cells excrete the bimane derivative of glutathione (GSMB) intact. *E. coli* (23), yeast (24), plants (25), and cultured mammalian cells (26, 27) all excrete GSMB produced within the cell into a vacuole or to the extracellular space using an ATP-requiring ABC transporter. Thus, the intracellular degradation of MSmB in mycobacteria represents a marked departure from the pattern found in GSH-producing organisms.

The amidase is highly specific for the mycothiol moiety, lacking significant activity if either the acetyl or inositol residues are removed from MSmB to produce the substrates CySmB-GlcN-Ins or AcCySmB-GlcN, respectively (Figure 3). This demonstrates that the amidase is not a broad spectrum amidase but is highly specific for mycothiol derivatives, as expected if it plays a key role in a general mycothiol-dependent detoxification pathway.

Significant amidase activity was observed for a variety of groups attached to sulfur in the S-conjugate (Figure 4). Low but measurable activity was obtained with the two carbon acetamido moiety attached to sulfur (Figure 4, MSA) from reaction with iodoacetamide, whereas 4-fold greater activity was measured with an *N*-ethylsuccinimidyl residue (MSME) produced by reaction with *N*-ethylmaleimide. However, substantially larger maleimide derivatives (MSMC and MSMPB) exhibited only modestly increased activity. It is evident that the amidase can accommodate rather large groups attached to the sulfur. Nevertheless, MSmB was the best of the substrates tested, and modification of the bimane by attachment of a positively charged trimethylammonio group (MSqB) or a negatively charged *p*-sulfo benzoyloxy residue (MSsB) led to a 6–7-fold loss of activity (Figure 4).

The only S-conjugate studied that would occur in nature is that derived from cerulenin (MSC), an antibiotic produced by the actinomycete *Cephalosporium cerulens* (16). This mycothiol conjugate exhibited 8% of the activity found with MSmB. The S-conjugate structure shown in Figure 4 was drawn on the assumption that the reaction of cerulenin with mycothiol produces a product analogous to that demonstrated previously for its reaction with cysteine (28). On the basis of the finding that MSC is a reasonable substrate for the amidase, we propose that the amidase functions as a component of a mycothiol-dependent detoxification system in mycobacteria that can operate to inactivate bacterial antibiotics. In this regard, it may be significant that an *M. smegmatis* mutant blocked in mycothiol production, and thus lacking the amidase cofactor, was found to have 20-fold increased sensitivity to rifampin (3).

The amidase has little activity with mycothiol or mycothiol disulfide (Figure 3), which is an essential specificity to minimize a futile cycle involving amidase degradation of mycothiol or mycothiol disulfide in combination with mycothiol biosynthesis. Although mycothiol is not a substrate for the amidase at millimolar levels, it does inhibit amidase activity with MSmB as substrate.

The thiol biotinylating reagent 3-(*N*-maleimidopropionyl)-biocytin (MPB) is utilized to capture mycothiol as the

MSMPB conjugate in our current immunoassay protocols for determination of mycothiol (29). Since MSMPB is a substrate for the amidase, it is important that the amidase be inactivated when assaying cells by use of protein denaturing conditions for cell extraction as employed here and in the earlier study (29).

The amidase is an important practical tool for studies of mycothiol biochemistry because it provides an efficient means for producing GlcN-Ins. GlcN-Ins is required as a substrate for the assay of ATP-dependent cysteine/GlcN-Ins ligase (12, 17), as a standard for HPLC calibration (12), and as a precursor of synthetic analogues. Mycothiol is easily isolated from *M. smegmatis* and can be converted quantitatively to MSmB in minutes. Subsequent treatment with purified amidase produces an easily separated mixture of AcCySmB and stereochemically pure GlcN-Ins. This method is much faster and cheaper than the low yield isolation from *Micromonospora echinospora* (12) or the multistep chemical synthesis (17) previously used to produce GlcN-Ins.

Except for the first amino acid residue, the amino-terminal sequence for *M. smegmatis* amidase was identical to that of an open reading frame Rv1082 of the *M. tuberculosis* genome (20). Confirmation that this gene codes for the amidase was obtained by showing that cloning and expression of Rv1082 in *E. coli* leads to the appearance of amidase activity in cell extracts. Open reading frames coding the identical amino-terminal sequence were also found in the reported sequences for *M. tuberculosis* CSU#93, *M. leprae*, *M. avium*, and *M. bovis*. Thus, genes coding for mycothiol S-conjugate amidase appear to be present in all of the mycobacterial genomes presently available, and it seems likely that homologues will be found in other mycothiol-producing actinomycetes. We propose the designation *mca* for the mycothiol S-conjugate amidase gene.

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