Published in final edited form as:

Curr Opin Chem Biol. 2009 October ; 13(4): 444–452. doi:10.1016/j.cbpa.2009.07.018.

Structures and Mechanisms of the Mycothiol Biosynthetic Enzymes

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Abstract

In the last decade, the genes encoding all four enzymes responsible for the biosynthesis of mycothiol in *Mycobacterium tuberculosis* have been identified. Orthologues of each of these have been stably expressed and structurally characterized. The chemical mechanisms of all four have also been studied. Due to the unique phylogenetic distribution of mycothiol, and the enzymes responsible for its biosynthesis, these enzymes represent interesting potential targets for anti mycobacterial agents.

Actinomycetes, including streptomycetes and mycobacteria species, produce mycothiol (MSH) instead of glutathione [1]. MSH is a redox-active low molecular weight thiol, formed by a conjugate of *N*-acetylcysteine (AcCys) with 1-*D*-myo-inositol-2-amido-2-deoxy- α -*D*-glucopyranoside (Gln-Ins) [2], as shown in Scheme 1. Mycobacteria produce the highest intracellular levels of MSH among actinomycetes [2]. Studies have shown that *Mycobacterium smegmatis* mutants lacking MSH become more sensitive towards oxidizing agents, electrophiles, and antibiotics [1,3,4], suggesting a critical role of MSH in the survival and pathogenicity of mycobacteria. A mechanism of detoxification of electrophilic toxins by MSH has been proposed (Scheme 1, path a). MSH reacts to form an MSH toxin *S*-conjugate that is subsequently hydrolyzed by the mycothiol *S*-conjugate amidase (Mca), yielding a mercapturic acid and Gln-Ins [3]. The mercapturic acid is transported out of the cell, whereas the other product, Gln-Ins, remains in the cell for the re-synthesis of MSH (Scheme 1, path b). In addition to its role as an intracellular redox buffer, interactions were recently observed in *Streptomyces coelicolor* between MSH and the transcription system, indicating that MSH serves as a modulator of the transcription system for its own production and consumption [5].

The biosynthesis of MSH is a five-step process that involves four unique enzymes, MshA, MshB, MshC, and MshD, as illustrated in Scheme 1, path b. The process is initiated by an *N*-acetyl-glucosamine transferase (MshA) to generate 3-phospho-GlcNAc-Ins, which is subsequently dephosphorylated to generate GlcNAc-Ins by an unknown phosphatase [6]. GlcNAc-Ins is deacetylated by MshB [7], and the resulting Gln-Ins is ligated to *L*-cysteine by MshC [8]. The Cys-Gln-Ins is then acetylated by MshD, yielding the final product, which we term MSH. Mutant strains of *M. smegmatis* with modifications, mutations or deletions in the genes encoding MshA-D have been investigated for MSH production and drug susceptibility [4,9-11]. Since the MSH pathway is not found in eukaryotes or other eubacteria, the enzymes

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involved in MSH biosynthesis present potential and *specific* chemotherapeutic targets for agents with activity against Actinomycetes, including mycobacteria.

Rational lead inhibitor design requires detailed biochemical, mechanistic and structural characterization. Towards this goal, significant progress has been made with the four mycothiol biosynthetic enzymes, MshA-D, in this decade. With the recently reported structures of MshA and MshC [12,13], the crystal structures of all four of these key enzymes have been determined [14-16]. Here we report and summarize the current mechanistic, kinetic, and structural characteristics of the enzymes of mycothiol biosynthesis.

The glycosyltransferase, MshA

The first step in the biosynthesis of mycothiol is catalyzed by the glycosyltransferase MshA. The enzyme catalyzes the formation of 3-phospho-1-D-*myo*-inosityl-2-acetamido-2-deoxy- α -D-glucopyranoside by transferring *N*-acetylglucosamine from UDP-*N*-acetylglucosamine (UDP-GlcNAc) to 1-*L*-*myo*-inositol-1-phosphate (1-*L*-Ins-1-P). The anomeric configuration of the transferred GlcNAc moiety is retained in the product thus identifying MshA as a retaining glycosyltransferase. This activity was first characterized in crude lysates of *M. smegmatis* [6]. More recently, we have reported the structural and kinetic description of the purified, recombinant enzyme from *Corynebacterium glutamicum* [13]. Initial velocity studies are consistent with a sequential kinetic mechanism.

The three-dimensional crystal structure demonstrates that MshA belongs to the GT-4 family of glycosyltransferase enzymes [13] and has a GT-B fold (Figure 1A), as predicted from the protein sequence [17]. The GT-B fold consists of two $\beta/\alpha/\beta$ Rossmann-fold domains connected by a short hinge region. MshA crystallized as a dimer, consistent with solution studies, with the dimer interface located in the *N*-terminal domain. In the apo-form, each monomer adopts an unusually extended conformation with the binding sites for UDP-GlcNAc and 1-*L*-Ins-1-P distant in space. In the structure of the MshA-UDP complex, MshA exhibits a closed conformation (similar to that exhibited by other GT-4 family members) with UDP bound to the *C*-terminal domain. The closed structure is a consequence of a 97° rotation of the *C*-terminal domain relative to the *N*-terminal domain and results in the formation of the binding pocket for 1-*L*-Ins-1-P. Combined with the kinetic studies, this structural result suggests that MshA utilizes an ordered sequential mechanism with UDP-GlcNAc binding first, followed by 1-*L*-Ins-1-P.

Based on structures of related glycosyltransferase enzymes with the intact sugar nucleotide bound [18,19], a model of the ternary complex was constructed where the GlcNAc moiety binds in a bent-back confirmation forming interactions with the diphosphate moiety (Figure 1B). This model allowed us to further analyze possible enzyme mechanisms. A double-displacement mechanism, similar to the mechanism utilized by the retaining glycosidase lysozyme [20], is not likely due to the lack of a readily identifiable nucleophile in the active site of MshA, as well as in structures of similar retaining glycosyltransferase enzymes [21]. Instead, an alternative S_Ni mechanism [22] was proposed where nucleophilic attack by the hydroxyl of 1-*L*-Ins-1-P and departure of UDP occurs on the same face, resulting in retention of anomeric configuration (Figure 1C). The transition state of this highly dissociative mechanism would have significant oxocarbenium ion character. Additionally, the β -phosphate of UDP-GlcNAc is proposed to abstract a proton from the 3-hydroxyl of 1-*L*-Ins-1-P, providing an example of substrate-assisted catalysis.

The deacetylase, MshB

MshB was identified in a genetic analysis of a selected MSH-deficient mutant strain of *M. smegmatis*. This strain did not produce GlcNAc-Ins, but could utilize added GlcNAc-Ins from

the medium for the synthesis of MSH [7]. In this study the deacetylase activity of MshB was demonstrated and shown to be encoded by the Rv1170 gene in *M. tuberculosis*, making this the first gene identified in the mycothiol biosynthesis pathway. MshB is sequence-related to Mca, the enzyme involved in electrophilic toxin elimination [3]. MshB is not essential for MSH synthesis or growth of *M. tuberculosis*, since Mca has been shown to have a low GlcNAc-Ins deacetylase activity that enables production of MSH in the absence of MshB [23].

MshB from *M. tuberculosis* is a Zn^{2+} -dependent metallohydrolase [15,16]. Structural studies revealed that the N-terminal domain of MshB resembles the fold of lactate dehydrogenase, with an α/β fold C-terminal domain (Figure 2A). The catalytic Zn^{2+} atom is located in the N-terminal domain, and is coordinated by His13, Asp16, His147 (Figure 2B) along with two water molecules, one of which is likely to be displaced upon the binding GlcNAc-Ins [15]. The crystal structure obtained with β -octylglucoside identified the putative active site as a cavity enclosed under C-terminally located loops [16]. This was further supported with the observed coordination of a mercuric acetate ion, used in the structural determination, by the Zn^{2+} ligands.

A catalytic mechanism was proposed for MshB based on the similar structural arrangement of catalytic residues observed in metalloproteinases [15]. The conserved Asp15 was suggested to be the general base for water activation in this mechanism [15]. As illustrated in Figure 2C, catalysis is initiated by proton abstraction of the zinc-ligated water molecule by Asp15. The deprotonated water then acts as a nucleophile to attack the amide carbonyl, which is polarized by the zinc. The resulting tetrahedral transition state would be stabilized by the positively charged Zn^{2+} and the side chain of His144, which is hydrogen bonded to Asp146. The carboxyl group of Asp15 acts as a general acid for the proton transfer to the amine, releasing GlcN-Ins as product. However, more detailed mechanistic studies are needed to validate this hypothesis.

The substrate specificity of MshB has been investigated and compared to Mca [24]. An overlap of substrate specificity was observed for the two enzymes. In general, MshB is a better deacetylase than Mca with the exception of the monobimane derivative, CysmB-GlcNAc-Ins. CysmB-GlcNAc-Ins is a better substrate towards the amidase activity of MshB than its natural N-acetylated substrate. Recently, MshB was screened with a library of natural bromotyrosine-derived compounds containing a spiroisooxazoline moiety that were previously shown to inhibit Mca. Although the inhibitory concentrations of the best inhibitors remain in the low micromolar range, these results provide the basis for further development and optimization of more potent inhibitors [25].

The ligase, MshC

MshC catalyzes the ATP-dependent condensation of cysteine and GlcN-Ins. MshC was shown to be essential for production of MSH in *M. smegmatis* [4]. In *M. tuberculosis*, the *mshC* gene has been demonstrated to be essential for *in vitro* growth [26,27]. MshC was initially identified by Sareen et al. from crude extracts of *M. smegmatis* [28] and the ligase activity was demonstrated in crude extracts and the N-terminal sequence was determined. This allowed the gene encoding the *M. tuberculosis mshC* gene to be identified and revealed that MshC shares primary sequence, functional and, presumably, structural similarities with cysteinyl-tRNA synthetase (CysRS) based on a sequence identity of 37.6% [28]. Significant difficulties have been encountered with efforts to purify *M. tuberculosis* MshC [29].

MshC from *M. smegmatis* was recombinantly expressed, purified and characterized [30]. The enzyme is a monomer with a molecular weight of ~ 47 kDa. The steady state kinetics along with positional isotope exchange (PIX) experiments demonstrated that the reaction catalyzed by MshC follows a Bi Uni Uni Bi Ping Pong mechanism (Figure 3C), with the random binding of ATP and L-cysteine, release of pyrophosphate, binding of GlcN-Ins and finally the release of

Cys-Gln-Ins and AMP [30,31]. A stable bisubstrate analog of the adenylate intermediate, 5'-O-[N-(L-cysteiny)sulfamonyl]adenosine, CSA, exhibits competitive inhibition versus ATP ($K_i \sim 300$ nM) and non-competitive inhibition versus cysteine. On this basis, the overall reaction can be divided into two steps: cysteine adenylation and the subsequent ligation of cysteine to Gln-Ins [30]. Single-turnover studies of the first and second half reactions supported the cysteinyl-adenylate as a kinetically competent intermediate in the reaction by MshC [30, 31].

The inability to crystallize the full length *M. smegmatis* MshC was overcome by incubation of the enzyme with the tight binding inhibitor, CSA, followed by a 24-hour limited trypsin proteolysis. The resulting enzyme was successfully crystallized and the structure solved, providing the three-dimensional structure of MshC with CSA bound in the active site at 1.6 Å resolution [12]. The refined structure exhibited electron density for all MshC residues, except residues 285 – 297, which presumably form a flexible active site loop structure that is proteolyzed. The overall tertiary fold is similar to that of cysteinyl-tRNA synthetase [32], with a Rossmann fold catalytic domain (Figure 3A). The structure also revealed that the bound zinc ion forms a direct interaction with the ligand thiolate at the base of the active site, which may serve a primary function in amino acid discrimination. A number of completely conserved active site residues (Figure 3B) were observed to interact with the ligand, providing guidelines for site-directed mutagenesis studies. The OG1 sidechain oxygens of T46 and T83 form hydrogen bonds with the cysteine moiety of CSA. The 2'-oxygen of the ATP moiety makes a 2.7 Å hydrogen bond with the D251 side chain carboxylate group. The conserved H55 of the HxGH motif is 3.4 Å away from the ribose oxygen atom in the MshC-CSA complex, but it is likely that a hydrogen bond would form in the structure with the cysteinyl adenylate intermediate. The indole ring nitrogen of W227, proposed to be responsible for amino acid discrimination and positioning in CysRS [32], is 3.3 Å away from the thiol group of cysteine, and likely forms a hydrogen bond with the cysteine thiolate in the adenylation reaction. To assess the functional roles of these residues in substrate binding and catalysis, we have constructed the following mutant forms of the enzyme: T46V, H55A, T83V, D251A, D251N, W227F and W227H. The results of these studies have assisted in defining the catalytic mechanism and residues that contribute to substrate binding and catalysis (Fan F. and Blanchard, J. S., in press).

The acetylase, MshD

MshD catalyses the final step in mycothiol biosynthesis, the transfer of an acetyl group from acetyl-CoA to the amino group of Cys-Gln-Ins to yield MSH. The structure of *M. tuberculosis* MshD was determined in a binary complex with AcCoA and ternary complex with CoA and desacetylmicothiol (DAM) to a resolution of 1.7 and 1.8 Å, respectively [14, 33]. MshD is composed of two GCN5-related N-acetyl transferase domains (GNAT), an N-terminal domain (1-140) and a C-terminal domain (151-315) connected by a random coil linker (Figure 4A). GNATs most frequently occur as dimers where terminal β-strands from the core β-sheet of each monomer interact to form a continuous β-sheet across the dimer interface [34]. MshD is a fused version of this arrangement with the general orientation of the two GNAT domains similar to other dimeric family members. The two structural features utilized by GNAT family members to coordinate their common ligand, acetyl-Coenzyme A (AcCoA), are: i) a loop between β4 and α3 that positions several amide backbone atoms in a similar direction and is utilized to coordinate the pyrophosphate moiety and ii) a splay between β-strands 4 and 5 of the core β-sheet which is utilized to coordinate the peptide-like character of the β-alanyl pantetheine moiety [34]. Both GNAT domains of MshD were found to coordinate AcCoA through these features, however it was proposed that only the C-terminal domain was biologically active with the N-terminally bound AcCoA utilized as a structural element for stability [14]. In addition, the central cavity between the two domains appeared to be larger

than required to bind the acceptor substrate, DAM, suggesting there may be domain movement upon substrate binding. The structure of the ternary complex with CoA and DAM confirmed many of the hypotheses proposed from the binary AcCoA complex [33]. A single molecule of DAM binds in the central groove between the two GNAT domains with the amine group adjacent to the cofactor bound to the C-terminal domain. In addition, the N-terminal domain retains a bound AcCoA molecule despite high concentrations of acceptor substrate. This confirms that the C-terminal domain is the active domain and that the N-terminally bound cofactor does not exchange once the protein has folded. In the ternary complex, the domains clamp down on DAM with protein atoms from both domains utilized to coordinate the ligand. Submission of the binary and ternary structures of MshD to protein domain motion analysis within the program DYNDOM [35], indicates that there is a 17.9° rotation of the N-terminal domain relative to the C-terminal domain with the central β -sheet residues 131-133 and 303-304 acting as hinge residues (Figure 4B). Enzymatic analysis suggests that MshD catalyzes the direct transfer of the acetyl group to desacetylmethylthiol without use of an enzyme bound acyl-intermediate. Additionally, MshD exhibits a bell shaped pH dependence on $V_{\max}$ and V/K_{DAM} suggesting a catalytic base (pK_a ranges from 6.6 to 7.0) and a catalytic acid (8.7-9.2) participate in the reaction. The $V_{\max}$ was calculated as $8.3 \pm 0.6 \text{ s}^{-1}$ and the K_m values for acetyl-CoA and Cys-GlcN-Ins were determined to be $40 \pm 5 \text{ }\mu\text{M}$ and $82 \pm 22 \text{ }\mu\text{M}$, respectively. The structure of the binary and ternary complex is consistent with the enzymatic analysis, as the amine of desacetylmethylthiol was found to be correctly positioned for in-line attack on the re-face of the acetyl group of AcCoA [33] (Figure 4C). The side chains of Glu234 and Tyr294 were determined to be in a suitable position to act as the general base and acid, respectively, in the reaction [33].

Conclusions

MSH plays important roles in the survival and pathogenicity of mycobacterial species, including *M. tuberculosis*, by allowing the organism to cope with a broad spectrum of oxidants, electrophiles, as well as a number of antibiotics [3]. Since the MSH biosynthetic pathway is not present in eukaryotic cells, the enzymes involved in MSH biosynthesis present appealing antimicrobial targets. Mutant *M. smegmatis* strains with modification, mutation or deletions in the genes encoding MshA-D were investigated for MSH production and drug resistance [4,9-11]. Genetic disruptions of *mshA* in *M. tuberculosis* have produced strain-dependent results with *mshA* being reported as essential for growth in the Erdman strain [36], but nonessential in the H37Rv strain [37]. Disruption of *mshB* resulted in a 90-95% decrease in MSH production. However, this mutant did not show significant differences in sensitivity towards most oxidative and alkylating stress, as well as antibiotics, indicating that the low amount of MSH present in the mutant is sufficient to protect the cells against stress [11]. Intriguingly, both *MshA*⁻ and *MshB*⁻ mutant strains were observed to be ~6 folds more resistant towards ethionamide compared to the wild type strain [11]. Knockout mutants of *M. tuberculosis* MshD resulted in a dramatic increase in cellular levels of Cys-GlcN-Ins, very low levels of MSH (by way of a direct cellular reaction of Cys-GlcN-Ins with acetyl-CoA), and a substantial level of (formyl)Cys-GlcN-Ins which acts as a poor surrogate for MSH [38,39]. The phenotype of the knockout mutant suggested that MshD was non-essential in rich media, but reasonably essential in times of oxidative stress. In contrast, studies with a *MshC*⁻ mutant shown to have ~ 80 fold less activity than wild type MshC, exhibited significantly increased susceptibilities to oxidative and alkylating stress, as well as a variety of antibiotics [4]. The *MshC*⁻ mutant was also more sensitive to ethionamide [4]. These studies indicate that MshA and MshC are more promising drug targets towards conventional antibiotic treatment over other MSH biosynthetic enzymes.

A number of research groups have carried out a detailed molecular, biochemical, mechanistic as well as structural characterizations of MSH biosynthetic enzymes. In the past decade,

significant progress has been made, with the genes of all key enzymes identified, proteins purified in recombinant forms, and structures solved to high resolution, yielding an in-depth understanding of MSH metabolism and bioremediation. These data will enhance our ability to synthesize useful compounds that selectively target MSH biosynthetic pathway in several human pathogens.

Acknowledgments

We thank Dr. Gavin Painter and group (Gracefield Research Center, New Zealand) for the synthesis of GlcN-Ins, and Lee W. Tremblay for helpful discussions.

This work was supported in part by a grant from the National Institute of Health (AI33696 to J. S. B.), and a Fellowship from the Heiser Program for Research in Leprosy and Tuberculosis of The New York Community Trust to F. F., and a Fellowship from the Charles H. Revson Foundation to P. A. F.

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Figure 1.

A) Three-dimensional structure of MshA monomer (PDB_{1D} 3c4v). **B)** Stereo stick diagram of ternary complex model. 1L-Ins-1-P carbons colored yellow, UDP-GlcNAc carbons colored green. Important hydrogen bonding interactions shown as grey dotted lines, and the interaction of the 3-OH of 1L-Ins-1-P with the UDP-GlcNAc at the site of reaction chemistry shown as cyan dotted lines. **C)** Proposed S_Ni – substrate assisted catalysis mechanism, featuring oxycarbenium-ion like transition state, with asymmetric phosphor bond breakage, and glycosidic bond formation.

Figure 2.

A) Three-dimensional structure of MshB monomer (PDB_{ID} 1q74). **B)** Stereo stick diagram enzyme active site of MshB, with the catalytically relevant zinc atom, coordinated by His13, Asp16 and His146 along with the two water molecules. **C)** Proposed catalytic mechanism for MshB. In this mechanism, the highly conserved Asp15 acts as general base to deprotonate the active site water molecule, which in turn carries out a nucleophilic attack to the amide carbonyl polarized by the zinc atom.

Figure 3.

A) Three-dimensional structure of MshC monomer (PDB_{ID} 3c8z), resolved at 1.6 Å. **B)** A diagram illustrating the active site of MshC, in presence of tight binding inhibitor CSA. **C)** The proposed Bi Uni Uni Bi Ping Pong kinetic mechanism of MshC, based on steady state kinetic as well as PIX studies.

Figure 4. Structure and mechanism of MshD. A) Ribbon diagram of the CoA-DAM-MshD ternary complex. Ligands shown as sticks colored by atom type. B) DYNDOM analysis diagram showing the domain movements upon formation of the ternary complex. The MshD-AcCoA complex (open; PDB_{ID} 1ozp) shown as green trace, while the MshD-CoA-DAM ternary complex (closed; PDB_{ID} 2c27) shown with the N-terminal domain in cyan and the C-terminal domain in blue. The hinge residues are colored red, and the orange arrow is the axis of the domain rotation. $\alpha 2$ is colored magenta in both the open and closed conformation as a reference point. The ligands for the MshD-AcCoA complex and the AcCoA bound to the N-terminal domain are omitted for clarity. C) Direct attack of the desacetylmycothiol amine upon the acetyl

group of AcCoA. Chemistry is facilitated by the partial charge of the backbone amide of Leu238 stabilizing the tetrahedral intermediate and Glu324 and Tyr294 acting as active site base and acid, respectively.