The Substrate-induced Conformational Change of Mycobacterium tuberculosis Mycothiol Synthase*§

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The structure of the ternary complex of mycothiol synthase from Mycobacterium tuberculosis with bound desacetylmycothiol and CoA was determined to 1.8 Å resolution. The structure of the acetyl-CoA-binary complex had shown an active site groove that was several times larger than its substrate. The structure of the ternary complex reveals that mycothiol synthase undergoes a large conformational change in which the two acetyltransferase domains are brought together through shared interactions with the functional groups of desacetylmycothiol, thereby decreasing the size of this large central groove. A comparison of the binary and ternary structures illustrates many of the features that promote catalysis. Desacetylmycothiol is positioned with its primary amine in close proximity and in the proper orientation for direct nucleophilic attack on the si-face of the acetyl group of acetyl-CoA. Glu-234 and Tyr-294 are positioned to act as a general base and general acid to promote acetyl transfer. In addition, this structure provides further evidence that the N-terminal acetyltransferase domain no longer has enzymatic activity and is vestigial in nature.

The importance of mycothiol to mycobacterial organisms and the absence of mycothiol in eukaryotes and instead produce mycothiol (acetyl-L-cysteine-1-D-myo-inosityl-2-deoxy-D-glucopyranoside) (1, 2). Similar to glutathione, mycothiol provides the cell protection from oxidative damage and electrophilic toxins (3, 4) and is maintained in the reduced state by a reductase (5). Mycothiol is less prone to metal ion-catalyzed autoxidation than is glutathione and may serve as a stable intracellular store of cysteine (1). Mycobacterium smegmatis mutants that were deficient in mycothiol production were hypersensitive to alkylating agents, free radicals, and antibiotics (4, 6). Mycobacteria produce a especially large amount of mycothiol, with the intercellular concentration in Mycobacterium tuberculosis reaching millimolar concentrations (7).

The protection of the primary amine by acetylation is therefore presumed to be an important step in the formation of a useful detoxification agent.

The previously reported structure of the MshD-AcCoA binary complex demonstrated that MshD belongs to the GCN5-related family of N-acetyltransferases (GNATS) (10). In general, GNATS catalyze the transfer of an activated acetyl group from AcCoA to the primary amine of the target, be it a protein residue (i.e. αN termini or lysine) or small molecule (i.e. spermine, serotonin, aminoglycoside, glucosamine 6-phosphate, etc.) (11). The GNAT family also includes outliers such as the FEM family of N-acetyltransferases and N-myristoyltransferases, which utilize alternate activated substrates. Despite low sequence homology, GNATS with diverse substrates have similar overall tertiary structure. The structural similarity among GNATS is determined by their need to bind the common substrate, AcCoA, whereas the total lack of sequence homology is a consequence of the manner in which AcCoA is coordinated, as most of the interactions are through backbone atoms. Indeed, the main feature of the GNAT fold is the generation of the AcCoA binding site. One element of this site is the incomplete adjoining of two β-sheets, thereby forming a single β-sheet with a V-like appearance. The exposed β-sheet backbone amides and carbonyls are used to coordinate the β-alanyl pantetheine backbone of AcCoA. A second common element is the “P-loop,” a loop connecting two conserved pieces of secondary structure that typically points several consecutive amide backbone atoms in a similar direction to coordinate the oxygen atoms of pyrophosphate. The adenine moiety of CoA is typically solvent-exposed and does not contribute significantly to the interaction with the GNATS. Variable side chains in a 10–15 Å sphere around the activated acyl group are then used to interact with the target substrate and provide substrate specificity.

Many of the GNATS from prokaryotes are dimeric in solution, and often, the active site is located at the dimer interface such that groups from both monomers of the dimer contribute to the active site architecture (11, 12). MshD, however, is a monomer in solution, but interestingly, it contains two GNAT domains (10). The N-terminal GNAT domain (1–140) and the C-terminal GNAT domain (151–315) are joined by a random coil linker (141–150) with the relative orientation of the two GNAT domains similar to many other dimeric GNAT proteins.

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† The atomic coordinates and structure factors (code 2C27) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: MshD, mycothiol synthase; DAM, desacetylmycothiol; CoA, coenzyme A; AcCoA, acetyl-coenzyme A; GNAT, GCN5-related N-acetyltransferase; r.m.s.d., root mean squared deviation; Bicine, N,N-bis(2-hydroxyethyl)glycine.
SCHEME 1. Biosynthesis and reactions of mycothiol. Mycothiol is synthesized in four consecutive steps catalyzed by MshA–D. Oxidation yields the disulfide, mycothione (M-S-S-M), which can be reduced by the flavoprotein disulfide reductase, mycothione reductase. Reaction of mycothiol with electrophiles generates the S-alkylmycothiol, which can be cleaved by the amidase to generate the mercapturic acid and the pseudodisaccharide that is a substrate for MshC.
The domains share a similar topology, exhibiting an r.m.s.d. of 1.7 Å over 88 structurally similar residues and are believed to have arisen from a gene duplication and fusion event.

Although MshD is composed of two GNAT domains, and each is competent to bind a molecule of AcCoA, the structure of MshD in complex with AcCoA and CoA suggested that only the C-terminal GNAT domain was active (10). Modeling of DAM in the active site groove between the two GNAT domains revealed that the groove was much larger than needed for binding, indicating that there may be some domain movement upon binding substrate (10). Unfortunately, the malleability of the GNAT active site residues, possible domain movements upon binding substrates, and unknown nature of the two MshD active sites make accurate modeling of DAM in the MshD active site very difficult. The design of inhibitors requires intimate details of the interactions of substrates with their active sites; therefore, a high resolution MshD ternary complex is highly desirable. Here we present the 1.8 Å resolution structure of MshD in complex with the disulfide of CoA and DAM, the detailed interactions of DAM and MshD residues, and the structural rearrangements upon complex formation and present further evidence that having two GNAT domains, only the C-terminal domain is catalytically active. This is the first structural report of a mycothiol biosynthesis enzyme in complex with its full ligand set.

**EXPERIMENTAL PROCEDURES**

**Purification and Measurement of Enzyme Activity—**Recombinant MshD was expressed and purified as reported previously (10). The synthesis of DAM was carried out as described for similar compounds (13). DAM was purified and characterized as the molecular disulfide (see Supplemental Materials). Reaction rates were determined by monitoring the decrease in absorbance at 232 nm due to hydrolysis of the thioether bond of acetyl-CoA or propionyl-CoA (ε = 4500 M⁻¹ cm⁻¹) in a Uvikon XL spectrophotometer equipped with thermostatic heaters and connected to a constant temperature, circulating water bath. Assays were performed in 100 mM K₂HPO₄, pH 7.0, and 100 μM Tris(2-carboxyethyl)phosphine hydrochloride at a temperature of 25 °C. Reactions were initiated by the addition of enzyme. For every enzymatic reaction, a corresponding non-enzymatic control reaction was carried out. The non-enzymatic rate was then subtracted from the enzymatic rate before fitting to Equation 1 using

\[
v = \frac{V_{AB}}{(K_{iA}K_B + K_AB + K_BA + AB)} \quad \text{(Eq. 1)}
\]

where \(v\) is the measured velocity, \(V\) is the maximal velocity, \(A\) and \(B\) are the concentrations of substrates \(A\) and \(B\), respectively, and \(K_{iA}\) and \(K_B\) are the Michaelis-Menten constants for substrates \(A\) and \(B\), respectively. Enzyme activity was measured over the pH range of 6.0–9.0 using the non-enzymatic rate before fitting to Equation 1 using

\[
v = \frac{VA}{(K_A + A)} \quad \text{(Eq. 2)}
\]

where \(v\) is the measured velocity, \(V\) is the maximal velocity, and \(A\) is the concentration of substrate \(A\). The \(V\) and \(K_A\) were determined using six concentrations of \(A\) with saturating concentration (–6× \(K_A\)) of \(B\). The pH dependence of \(V\) and \(V/K\) were then fitted to Equation 3.

\[
\log V \text{ or } V/K = \log[C/(1 + H^+/K_A + K_{H^+}/H^+)] \quad \text{(Eq. 3)}
\]

where \(C\) is the pH-independent plateau value, \(K_A\) is the ionization constant for the acidic group, \(K_{H^+}\) is the ionization constant for the basic group, and \(H^+\) is the hydrogen ion concentration.

**RESULTS AND DISCUSSION**

Crystallization and Structure Determination—Prior to crystallization setups, MshD (15 mg/ml, 20 mM triethanolamine, pH 8.0) was incubated with 7.7 mM DAM, 5.3 mM CoA, and 40 mM dithiothreitol for a period greater than 4 h at 4 °C. Crystallization was by vapor diffusion under oil in which 2 μl of the MshD-ternary complex was combined with 2 μl of crystallization solution (1 mM Na₃ citrate, pH 8.5, 100 mM Bicine, pH 8.8) under 100 μl of Fisher silicon oil. Crystallization trays were stored uncovered at 18 °C. Cube-shaped crystals belonging to the space group P2₁, with approximate unit cell dimensions of \(a = 37.8, b = 59.6, c = 61.7\) Å, and \(β = 91.6°\) were of diffraction quality in 2–4 days. This crystal form was not stable to cryoprotection and vitrification, so data were collected at room temperature. An MshD-ternary complex crystal was scooped directly from a crystallization drop and enclosed in a capillary using the method of Sweeny and D’Arcy (14). During the experiment, the crystal was in vapor equilibrium with 20 μl of mother liquor (1 mM Na₃ citrate, 100 mM Bicine, pH 8.8) positioned at the flame-sealed end of the capillary. Data were collected on an MSC R-Axis IV⁺ image plate detector using CuKα radiation from a Rigaku RU-H3R X-ray generator and processed using DENZO/SCALEPACK (15) (Table 1). The program AMORE (16) was used for molecular replacement (see “Results and Discussion”). Interactive model building utilized the molecular graphics program O (17) and was followed by refinement within CNS (18). The final model has an R-factor and R-free of 16.1 and 20.3, respectively, with good stereochemistry and geometry (Table 1).

Electron densities corresponding to residues 1–4, 56–58, 266–268, and 312–315 were not observed in the 2Fo – Fc maps and therefore were not modeled. Figs. 3, 4 (A, C, and D), and 5 were prepared using Pymol (19).

**RESULTS AND DISCUSSION**

Kinetics—Initial velocity experiments were performed by varying the concentrations of AcCoA and DAM and yielded a series of intersecting lines. This is diagnostic of a ternary complex mechanism in which both substrates must be present on the enzyme before catalysis can occur. This kinetic mechanism is universally observed for members of the GNAT superfamily of N-acetyltransferases (11). As seen in Fig. 1A, the data points are well fitted by Equation 1. The lower value of \(K_{iAcCoA}\) when compared with the \(K_{AcCoA}\) causes the lines to appear almost parallel. To confirm the intersecting nature of the initial velocity plot, propionyl-CoA was used as an alternative substrate. As observed in Fig. 1B, the nature of the intersecting initial velocity pattern was apparent when using propionyl-CoA. This argues for a chemical mechanism in
which direct acetyl transfer from AcCoA to DAM occurs, as opposed to intermediate transfer from AcCoA to the enzyme followed by subsequent acetyl transfer to DAM.

To assess potential catalytic groups involved in catalysis, we determined the pH dependence of the maximum velocity and $V/K$ value for DAM. As shown in Fig. 2, the pH dependence of both $V$ and $V/K_{DAM}$ are bell-shaped, and the experimental data are well fitted by Equation 3. Both $V$ and $V/K_{DAM}$ depend on the ionization behavior of two groups: one group that must be deprotonated for activity and that likely serves as a catalytic base with $pK$ value of 6.6–7.0 and a second group that must be protonated for activity and likely serves as a catalytic acid with $pK$ value of 8.7–9.2. We ascribe the groups observed in both the $V$ and the $V/K_{DAM}$ pH profiles to the same enzyme groups and will discuss their likely identity in the following sections.

Domain Conformational Change—Crystallization of MshD in complex with CoA and DAM led to a $P_2_1$ crystal form that was not observed for the binary CoA/AcCoA complexes. Phasing by molecular replacement using the binary MshD-AcCoA complex as the search model was unsuccessful. Interestingly, using a search model consisting of only a portion of the N-terminal domain (residues 6–120) resulted in two significant rotation/translation solutions. These could be used to reconstruct a starting model containing both GNAT domains that, when subjected to simulated annealing refinement, resulted in an initial $R$-factor and $R$-free of 33.7 and 37.1%. Previously, it was observed that the central canyon running between the two domains was significantly larger than that required for binding of the second substrate, and it was proposed that there may be domain movement upon formation of the ternary complex (10). Indeed, failure of molecular replacement using the entire MshD monomer was due to changes in the relative orientation of the two domains. As can be seen in Fig. 3 (A and B), the N-terminal GNAT domain, as a unit, rotates significantly toward the C-terminal domain such that the central groove becomes much narrower. For example, relative to superimposed C-terminal domains, the N-terminal domain $\alpha$-helices (a1–a4, Cα carbons) of the binary complex versus the ternary complex exhibit r.m.s.d. values of 5.5–8.3 Å$^2$. The pivot point and genesis of this flexibility are the extended central $\beta$-strands, $\beta_6$ and $\beta_6'$, which in the binary complex are the only direct interactions between the two GNAT domains. In essence, the central extended $\beta$-strands are used as a fulcrum to change the relative position of the N-terminal domain relative to the C-terminal domain. This movement results in several new interactions between the

![FIGURE 1. Initial velocity patterns of mycothiol synthase. A, acetylation reaction. The symbols represent the experimen
tally determined values, whereas the solid lines are the best fit value for Equation 1. $V_{max} (8.3 \pm 0.6 \text{ s}^{-1}), K_{AcCoA} (40 \pm 5 \mu M), K_{DAM} (82 \pm 22 \mu M)$, and $K_{DAM_{AcCoA}} (2.9 \pm 2.0 \mu M)$ are the determined parameters. DAM was varied (●, 12.5 μM ○, 2.5 μM ▼, 50 μM ▽, 100 μM ■, 300 μM ▲) at fixed levels (3.75, 5, 7.5, 12.5, 30, and 60 μM) of acetyl-CoA. B, propionylation reaction. $V_{max} (2.2 \pm 0.3 \text{ s}^{-1}), K_{Pyropropionyl-CoA} (110 \pm 23 \mu M), K_{DAM} (90 \pm 20 \mu M)$, and $K_{Pyropropionyl-CoA} (2.6 \pm 3.2 \mu M)$ are the determined parameters. DAM was varied (●, 7.5 μM ○, 15 μM ▼, 30 μM ▽, 60 μM ■, 120 μM ▲) at fixed levels (5, 10, 20, 40, and 80 μM) of propionyl-CoA.

![FIGURE 2. pH dependence of the mycothiol synthase reaction. Upper panel, pH dependence of $V$; lower panel, pH dependence of $V/K_{DAM}$. The symbols are experimentally determined values, whereas the smooth curve is a fit of the data to Equation 3 (see “Experimental Procedures”).]
two GNAT domains. The α2'/β4' loop rests against the β3/β4 loop, which was disordered in the binary complex but is ordered in the ternary complex. The side chain of Arg-71 (β3/β4 loop) forms two hydrogen bonds with the backbone carbonyl of Gly-181 (α2'/β4' loop) and a single hydrogen bond with the carbonyl of Pro-178 (α2'/β4' loop). New salt bridges are formed between Arg-131 (β6) and Glu-179 (α2'/β4' loop) and Arg-40 (α2) with Glu-194 (α2'/β4 loop), whereas Glu-190 (α2') is electrostatically complemented by the side chain of Arg-46 (α2/β4 loop). In addition, there are numerous interactions between the two domains that are mediated by the bound substrate (see below). These movements and new interactions do not significantly alter the core structures of the individual domains. The r.m.s.d. for the N-terminal GNAT domain is 0.33 Å over 117 common atoms, whereas for the C-terminal GNAT domain, the r.m.s.d. is 0.37 Å over 119 common residues. Although localized changes have previously been observed upon substrate binding in various GNAT proteins, especially in the α1/α2 loop, this is the first instance of a large global conformational change.

DAM Interactions—Unambiguous electron density was observed for DAM within the active site groove and adjacent to the CoA molecule of the C-terminal domain (Fig. 4A). The average thermal factor for DAM was 14.2, similar to protein atoms in its vicinity, suggesting a well ordered and full occupancy small molecule. Electron density for the cysteine sulfhydryl of DAM suggested that it was in two conformations, both being in a disulfide linkage with the sulfhydryl of CoA. It may be possible that the conformational change is an artifact of the binding of CoA-DAM as a disulfide. However, it is highly unlikely that any CoA-DAM disulfide was generated in solution prior to crystal formation since the crystallization conditions contained 40 mM dithiothreitol to generate and maintain monomeric DAM. The finding of a CoA-DAM disulfide in the active site of the ternary complex is most likely a function of the close proximity of the thiols of CoA and DAM within the active site, the growth and incubation of crystals at room temperature prior to data collection, and the accessibility of the thiols to oxidation but not reduction once the substrates are enclosed within the active site. Therefore, the disulfide is not thought to have driven the conformational change nor altered the interactions of DAM or CoA with the active site. The sulfhydryl of DAM could be modeled through a simple rotation into a small adjacent pocket created by the side chains of Gln-180, His-177, and Phe-174.
FIGURE 4. Interactions of DAM with MshD. A, simulated annealing Omit map contoured at 3σ with DAM and CoA omitted from refinement and map calculation. The adenosine 3-phosphate moiety projects out into solvent and has poor density indicative of multiple conformations. B, schematic illustration of the interactions of DAM with the active site residues of MshD. C, stereo diagram of ternary complex. Interactions between DAM, cofactor, and MshD. Residues within 4.5 Å of the ligands are shown. The DAM, protein residues, and waters are from the MshD-DAM-CoA ternary complex, whereas cofactor is modeled as AcCoA based on its position in the MshD-AcCoA binary complex. The sulfhydryl of DAM has been modeled with a different rotomer so as to not interact with the cofactor. Atoms are colored by atom type. Carbons are colored white for DAM, AcCoA, and residues from the N-terminal GNAT domain, whereas carbons are colored brown for residues from the C-terminal domain. Waters that are hydrogen-bonded to DAM are displayed as cyan spheres. D, proposed reaction scheme.
The active site groove has an overall negative character and is highly polar in nature, consistent with the binding of a highly polar substrate with a primary amine functional group. Prior to binding DAM, the active site groove was solvated by ~40 waters and was significantly larger than required for binding substrate. After binding DAM, the N-terminal domain rotates toward the C-terminal domain, collapses over the substrates, and forms new domain-domain interactions. The large interdomain groove becomes a narrow tunnel with entrances large enough for solvent molecules but not for exit of the substrate. The tunnel retains ~30 waters, which further mediate interactions between the two domains. The substrate makes 5 direct and 11 water-mediated hydrogen bonds with side chains and backbone atoms of MshD within the tunnel (Fig. 4, B and C). A large number of these interactions are with the C-terminal domain, but there are two direct (Glu-36N, Lys-34N) and two water-mediated (Glu-79OE2, Asn-37N) hydrogen bonds with residues from the N-terminal domain, promoting the collapse of the large groove around the substrates, thereby shielding the reaction coordinate from bulk solvent. In addition, there are van der Waals interactions between the rings of substrate and Leu-133, Tyr-236, and Trp-110 at the bottom of the tunnel and Tyr-282 and Glu-36 at the top of the tunnel. The numerous interactions of DAM with MshD position the cysteinyl group of DAM in close proximity to CoA. Utilizing the MshD-AcCoA binary complex structure to model the acetyl group in an MshD-DAM-AcCoA pre-reaction model complex demonstrates that the primary amine of DAM is correctly positioned for in-line attack on the si-face of the acetyl group of AcCoA (distance = 2.5 Å) (Fig. 4, C and D). Glu-234, through an intervening water molecule, could act as a general base, accepting protons from the amine of DAM. This is presumably the group whose ionization behavior is observed on the acidic limb of the VpH profile. The backbone amide of Leu-238, which is hydrogen-bonded to and helps position the acetyl group of AcCoA, would stabilize the tetrahedral intermediate. Tyr-294 is suitably positioned to act as a general acid to protonate the sulfhydryl of CoA after breakdown of this intermediate. This is presumably the group whose ionization behavior is observed on the basic limb of the VpH profile. The use of an Asp or Glu through intervening water molecules as the active site base, Tyr as the active site acid, and utilization of a backbone amide for stabilization of the tetrahedral intermediate is a common theme in many GNAT enzymes (11), and these groups (Glu-234, Tyr-294, and Leu-238) appear to be the active site residues that MshD uses to promote the acetylation of DAM.

**N-terminal GNAT Domain**—Previously, it was suggested from the binary AcCoA and CoA complexes that only the C-terminal domain had acetyltransferase activity (10). This was based on three observations. (i) Although both domains bound an AcCoA molecule, only the acetyl group of the C-terminally bound AcCoA was solvent-exposed, whereas the acetyl group of the N-terminally bound AcCoA was buried; (ii) incubation of the binary AcCoA-MshD complex with excess CoA was unable to replace the AcCoA bound in the N-terminal domain, suggesting that the N-terminally bound AcCoA molecule does not exchange freely with solvent; (iii) some of the hallmarks of the GNAT fold are missing in the N-terminal domain, including a β-bulge in β4 and recognizable acid and base groups that could participate in chemistry. However, these observations do not conclusively demonstrate non-reactivity of the N-terminally bound AcCoA. For example, the recently determined structure of the ribosomal Nα-acetyltransferase, RimL, demonstrated that a β-bulge in β4 was not required for acetyltransferase activity (20). In addition, the local structure of the active site may significantly change during substrate binding and reaction chemistry, allowing either catalysis at the N-terminal domain or release of the AcCoA. Fig. 5, shows a simulated annealing Omit map for the N-terminally bound cofactor bound to the N-terminal GNAT domain. AcCoA was omitted from the refinement and map calculation.

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