Allostery and Conformational Dynamics in cAMP-binding Acyltransferases*

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Background: Cyclic AMP allosterically modulates GNAT-like acyltransferases (KAT) in mycobacteria. Results: Structures of the protein from M. smegmatis and its mutants are described.

Conclusion: The divergent properties of KATs from M. tuberculosis and M. smegmatis are the result of differential conformational dynamics in the two proteins.

Significance: Apparently similar proteins show subtle differences in their structural features, having evolved based on the needs of the organism.

Mycobacteria harbor unique proteins that regulate protein lysine acylation in a cAMP-regulated manner. These lysine acyltransferases from Mycobacterium smegmatis (KATms) and Mycobacterium tuberculosis (KATmt) show distinctive biochemical properties in terms of cAMP binding affinity to the N-terminal cyclic nucleotide binding domain and allosteric activation of the C-terminal acyltransferase domain. Here we provide evidence for structural features in KATms that account for high affinity cAMP binding and elevated acetyltransferase activity in the absence of cAMP. Structure-guided mutational analysis converted KATms from a cAMP-regulated to a cAMP-dependent acetyltransferase and identified a unique asparagine residue in the acyltransferase domain of KATms that assists in the enzymatic reaction in the absence of a highly conserved glutamate residue seen in Gcn5-related N-acetyltransferase-like acyltransferases. Thus, we have identified mechanisms by which properties of similar proteins have diverged in two species of mycobacteria by modifications in amino acid sequence, which can dramatically alter the abundance of conformational states adopted by a protein.

It is now appreciated that protein molecules exist in an ensemble of different conformations at equilibrium, and this conformational diversity provides a mechanism that allows multifunctionality as well as regulation of the activity of a protein (1, 2). The presence of multiple domains in a protein further provides an opportunity for allosteric regulation whereby binding of a ligand to one domain can alter the relative ratios of different conformational states of the protein at equilibrium. This “conformational selection” induced by ligand binding can now be reflected in a change in protein activity (3). Therefore, an understanding of the structural features of similar proteins with distinct biochemical properties can provide a molecular appreciation of the means by which the properties of individual proteins are regulated by allosteric effectors.

Cyclic AMP serves as an allosteric regulator for a number of effector proteins with a variety of functions (4, 5). We have recently reported that cAMP can regulate the acylation of lysine residues in mycobacterial proteins by binding to unique cAMP-regulated acetyltransferases (6, 7). We called these proteins lysine (K) acyltransferases from Mycobacterium smegmatis (KATms)3 and Mycobacterium tuberculosis (KATmt). Cyclic AMP binds to a classical cyclic nucleotide binding (CNB) domain N-terminal to a GNAT-like acetyltransferase (AT) domain. The two domains are connected to each other by a long linker region of ~60 residues. Despite an overall similarity in primary amino acid sequence in the CNB domains, KATms has a high affinity for cAMP (100 nM), whereas KATmt shows a 1000-fold lower affinity for cAMP (100 μM). Additionally, the basal acetyltransferase activity of KATms is higher and is able to utilize acetyl coenzyme A (acetyl-CoA) to acetylate universal stress protein (USP) even in the absence of cAMP with a ~2-fold increase in activity in the presence of cAMP. In contrast, cAMP binding to KATmt is essential to allow acetylation of USP (6).

To understand the mechanistic basis of cAMP-regulated acetyltransferase activity, we utilized hydrogen deuterium exchange followed by mass spectrometry (MS) approaches and showed that cAMP binding to KATms alters the dynamics of

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3 The abbreviations used are: KATms, lysine acyltransferase from M. smegmatis; KATmt, lysine acyltransferase from M. tuberculosis; GNAT, Gcn5-related N-acetyltransferase; CNB, cyclic nucleotide binding; AT, acetyltransferase; USP, universal stress protein; Ni-NTA, nickel-nitrilotriacetic acid; 2-ME, 2-mercaptoethanol; Bis-Tris, 2-[bis(2-hydroxyethyl)aminoo]-2-(hydroxy)methyl)propane-1,3-diol; MD, molecular dynamics; r.m.s.d., root mean square deviation; KAT, lysine acyltransferase.
the cAMP binding domain and the interdomain linker (8). We suggested that the linker region assumes a different conformation in the active cAMP-bound state, promoting enhanced catalysis through relief of inhibition. It was not clear at that time whether cAMP binding drives this conformational change as would be expected in the induced fit model or whether cAMP binding shifts the equilibrium to favor the active state. Recently, the crystal structure of KATmt was solved in both the presence and absence of cAMP and revealed that in the absence of cAMP in the structure KATmt adopted an autoinhibited conformation (9). The conformation of KATmt in the absence of cAMP was completely refractory to allowing substrate access to the catalytic site in the AT domain, accounting for the undetectable basal acyltransferase activity in the absence of cAMP. In contrast, upon cyclic AMP binding, a large conformational rearrangement in KATmt releases this autoinhibition with the linker region assisting in inducing the cAMP-mediated conformational switch.

However, we remained intrigued by the significant activity shown by KATms in the absence of cAMP, suggesting that distinct structural features in KATms must account for the reduced extent of autoinhibition of acyltransferase activity in the absence of cAMP. In the current study, we determined the structure of apoKATms and mutants that are compromised in cAMP binding or acyltransferase activity. The structures we describe here, along with confirmatory mutational analysis, identify distinct features that account for the divergent activities of KATms and KATmt in the absence of cAMP. Moreover, structure-driven biochemical analysis elucidated the role of specific residues in the AT domain of KATms that allow acetylation of its substrate, USP, in the absence and presence of cAMP. Finally, molecular dynamics simulations further showed the importance of the distinct amino acid sequence of the flexible regions in the KATms structure that enable sampling of the conformers in the absence of cAMP. Therefore, we have provided a molecular understanding of the basis for the vastly different biochemical properties of KATmt and KATms.

**EXPERIMENTAL PROCEDURES**

All fine chemicals were from Sigma-Aldrich. Routine bacterial growth medium (Luria Bertani) was purchased from Colloids Impex (India). Middlebrook 7H9 base and 7H10 agar were obtained from BD Biosciences. Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from GE Healthcare. Oligonucleotide primers were synthesized by labs. Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from MBI Fermentas (Canada) or New England BioLabsImpex (India). Middlebrook 7H9 base and 7H10 agar were obtained from BD Biosciences. Restriction enzymes were from New England BioLabs. Restriction enzymes were from MBI Fermentas. Restriction enzymes were from New England BioLabs.

**Cloning and Mutagenesis**—Point mutations in MSMEG_5458 were generated by site-directed mutagenesis as described previously (10). pProMSMEG_5458 (6) was used as the template, and mutations were confirmed by sequencing (Macrogen).

**Purification of Wild-type and Mutant KATms**—Purification procedures were essentially as described earlier (6) with a few modifications. Cultures of Escherichia coli (either BL21(DE3) or SP850 cye cells) harboring the respective plasmids were grown in Terrific Broth, and protein expression was induced following addition of 0.4 mm isopropyl 1-thio-β-D-galactopyranosidase. The cell lysate was loaded on Ni-NTA resin (Qiagen), and the hexahistidine-tagged protein was eluted with 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 300 mM imidazole, 5 mM 2-mercaptoethanol (2-ME), and 10% glycerol. The eluted protein was dialyzed overnight at 4°C against 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 5 mM 2-ME, and 10% glycerol in the presence of tobacco etch virus protease to specifically cleave off the hexahistidine tag. Cleaved protein and tobacco etch virus protease were removed by passing the sample through an Ni-NTA column in 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 10 mM imidazole, 5 mM 2-ME, and 10% glycerol. For crystallization, the final purification step included gel filtration on a Superdex 200 column (10/300 GL, GE Healthcare) equilibrated in 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 5 mM 2-ME, and 5% glycerol. Purified proteins were stored at a final concentration of 30 mg/ml at −80°C. Se-Met-derivatized wild-type KATms was prepared and crystallized in a manner similar to the native protein.

**Crystallization, Data Collection, and Crystal Structure Determination**—Crystals were obtained using the hanging drop vapor diffusion technique at 20°C and with a crystallization drop size of 2 μl with equal volumes of protein:reservoir solution. KATms wild-type Se-Met crystals were grown using a reservoir solution of 0.2 M NaCl, 0.1 M Bis-Tris (pH 6.5), 0.03 M CaCl₂, 2% 1,5-diaminopentane dihydrochloride, and 19% PEG 3350. The mutant KATms_R95K formed crystals with a reservoir solution of 0.2 M NaCl, 0.1 M Bis-Tris (pH 6.5), 0.03 M MgCl₂, 1.7% 1,5-diaminopentane dihydrochloride, and 21–23% PEG 3350. The mutant KATms_E234A formed crystals with a reservoir solution of 0.2 M NaCl, 0.1 M Bis-Tris (pH 6.5), 0.03 M CaCl₂, 2% 1,5-diaminopentane dihydrochloride, and 21% PEG 3350. Several rounds of microseeding were needed to improve the crystals. All crystals were cryoprotected in 25–30% (v/v) ethylene glycol before freezing in liquid nitrogen. Crystals of Se-Met derivatives were additionally soaked with 3.2 mM HgCl₂ for 2–4 h before freezing.

Crystallization was attempted in the presence of cAMP and/or acetyl-CoA, but the crystals obtained diffracted poorly. Poor diffraction was also obtained after soaking KATms crystals with cAMP and/or acetyl-CoA.

All x-ray diffraction data sets were collected at the synchrotron Elettra (Trieste, Italy; beamline XRD) at the wavelengths 0.975 Å for the Se-Met derivative of KATms wild type and KATms_E234A mutant and 1.000 Å for KATms_R95K mutant. Diffraction data were processed and scaled using HKL2000 and Scalepack (11). Data statistics are presented in Table 1. Phases for Se-Met crystals were obtained using anomalous data and PHENIX (12). The initial model of KATms wild type was improved with PHENIX Autobuild (12) and Arp/Warp (13, 14) followed by cycles of manual building in O (15). Structures of the KATms mutants R95K and E234A were determined by molecular replacement with Phaser (16) using KATms wild-type structure as the search model followed by cycles of manual building in O. Refinement of all structures was carried out in REFMAC (14, 17). Refinement statistics are given in Table 1.
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**TABLE 1**
Crystallographic statistics
The number of crystals for each structure is one.

| Data collection | KATms_WT | KATms_R95K | KATms_E234A |
|-----------------|---------|-----------|-------------|
| Beamline        | XRD Elettra | XRD Elettra | XRD Elettra |
| Wavelength (Å)  | 0.975   | 1.000     | 0.975       |
| Space group     | P2_1/2_1 | P2_1/2_1  | P2_1/2_1    |
| Cell dimensions | a, b, c (Å) | Resolution range (Å) | High resolution shell (Å) |
|                 | 38.30, 82.75, 105.51 | 41.40–1.90 | 1.94–1.90 |
|                 | 90.0, 90.0, 90.0 | 44.20–2.00 | 0.102 (0.668)* |
|                 | 21.9 (3.8) | 26.1 (1.8) | 0.047 (0.648) |
| Completeness (%)| 99.3 (98.4) | 96.9 (96.6) | 99.7 (100.0) |
| Redundancy      | 12.8 (11.4) | 4.3 (3.8) | 12.7 (12.0) |

| Refinement | Rworked (%) | Rwilson (%) | Number of atoms (protein/water/ions) | Average B-factors (protein/water/ions) | r.m.s.d. bond length (Å) | r.m.s.d. bond angle (%) | Ramachandran most favored (%) |
|------------|-------------|-------------|-------------------------------------|--------------------------------------|--------------------------|-------------------------|-------------------------------|
|            | 20.6        | 26.3        | 2473/171/3                          | 55.3/49.1/59.4                      | 0.018                    | 1.9                      | 91.4                          |
|            | 22.0        | 27.6        | 2485/124/3                          | 58.3/52.8/60.3                      | 0.017                    | 1.8                      | 91.9                          |
|            | 20.0        | 26.3        | 2453/124/2                          | 56.0/45.8/57.1                      | 0.015                    | 1.7                      | 91.7                          |

*Values in parentheses are for highest resolution shell.

**RESULTS**

**Overall Structure of KATms**—The polypeptide chain of wild-type KATms contains 333 residues that fold into two major domains (Fig. 1A). The N-terminal CNB-like domain comprises residues Val¹–Phe¹⁵⁹, and the C-terminal AT domain comprises residues Asp⁶⁰³–Val¹⁶. The two domains are connected by residues Ile¹⁴⁰–Ala¹⁵⁰, which we have earlier called the linker peptide (6). The conserved AT domain is followed by figures were made in PyMOL. Protein Data Bank codes are listed in Table 1.

**Western Blot Analysis**—Protein samples were electrophoresed on a 13.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was incubated with primary antibody overnight at 4 °C. KATms and USP polyclonal antisera were generated in the laboratory (6) and used at a dilution of 1:5000. Anti-acetyllysine antibody was used at a dilution of 1:2500. Horseradish peroxidase-conjugated secondary antibody (GE Healthcare) was used and detected by enhanced chemiluminescence (Luminata Crescendo, Millipore).

**In Vitro Acetylation Assays**—Acetylation assays were carried out in a total reaction volume of 20 µl containing 25 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, and 100 µM acetyl-CoA. USP (2 µg) prepared as described (6) was used as a substrate. The reaction was initiated by adding 200 ng of KATms in the presence or absence of cAMP (10 µM). Reactions were carried out at 22 °C for 10 min, terminated by boiling in 4× SDS sample buffer (200 mM Tris/HCl (pH 6.8), 8% SDS, 40% glycerol, 4% 2-ME, 50 mM EDTA, and 0.08% bromphenol blue), and analyzed by immunoblotting with acetyllysine antibody and enhanced chemiluminescence.

Continuous assays were performed as described earlier (6). The assay reaction mixtures contained 0.2 mM NAD, 0.2 mM thiamine pyrophosphate, 5 mM MgCl₂, 1 mM DTT, 2.4 mM α-ketoglutarate, 50 µM acetyl-CoA, 50 µM USP, 0.03 unit of α-ketoglutarate dehydrogenase, 190 mM KATms, and 20 mM HEPES (pH 7.5) in a total volume of 100 µl. The reaction was initiated by the addition of USP. The rates were analyzed continuously for 10 min by measuring NADH production at 340 nm using a UV-visible spectrophotometer (TECAN Infinite Pro Series).

**Molecular Dynamics**—The atomic detail structural models of wild-type KATms_WT and mutant KATms_P170H were constructed by the molecular modeling program CHARMM (18) starting from the crystal structure of the wild-type protein. Residues of the KATms_WT that were not defined in the crystal structure were obtained from on-site energy minimization of the corresponding peptide chains while keeping the rest of the protein structure fixed. The protein was then solvated in an explicit water environment modeled by the TIP3P water model (19) with at least 10 Å padding in each direction from the protein, resulting in an orthogonal periodic box of 92 × 72 × 62 Å (42,448 atoms). In addition, 10 sodium ions were added to achieve electroneutrality of the system. The solvent molecules and the hydrogen atoms in the protein were relaxed by a 2000 step minimization with the backbone atoms restrained at the initial structure. After the relaxation, the system was gradually heated from 0 to 300 K in 200-ps molecular dynamics (MD) simulation in the NVT (constant number of particles, volume, and temperature) ensemble in the absence of any structural restraint. An additional 500-ps MD simulation was performed at constant pressure (1 bar) and at 300 K temperature (constant number of particles, pressure, and temperature (NPT)). At this point, the mutation P170H was introduced in the wild-type structure by replacing the side chain of Pro with that of His. Overlapping water molecules were removed before structural optimization of the mutation site. Subsequently, we carried out a 35-ns NPT MD simulation on both systems. All simulations were performed using the CHARMM22 force field (20), and the particle mesh Ewald method (21) was used to treat long range electrostatic interactions with a direct space cutoff of 12 Å.
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Comparison of KATms and KATmt—Polypeptide chains of KATms and KATmt each contain 333 amino acid residues with 60% identity, resulting in similar subdomain structures with high conservation of secondary structure elements (Fig. 2). A structural comparison aligning 277 Ca atoms between KATms and the apo form of KATmt (Protein Data Bank code 4AVA) (22) gave a root mean square deviation (r.m.s.d.) value of 2.5 Å. Although the structure of KATms_WT is generally similar to KATmt_apo, significant structural differences are seen in specific regions of the CNB and AT domains as described later as well as in the conformation of the linker peptide that influences the angle between the two domains (Fig. 1C). As expected, the KATms_WT_apo structure differs more profoundly from the cAMP-bound KATmt (Protein Data Bank code 4AVB) due to a large conformational change in the linker region of KATmt upon cAMP binding and thus the angle between CNB and AT domain but still retains a similar architecture of the individual CNB and AT domains (Fig. 1D).

Structural Features of the CNB Domain and cAMP Binding Site—The extreme N terminus is more structured in KATms than in KATmt with a significantly longer α1 helix (Figs. 1C and 2). The polypeptide chains start to structurally overlap at residues Ala9 and Val12 in KATms and KATmt_apo, respectively. An important structural deviation is seen in the loop between helix α1 and helix α2 probably because of Pro32, a residue that is present only in KATms, whereas other mycobacterial orthologs have an alanine at this position (6).

A second and significant difference between KATms and KATmt structures is located at the β3-turn-β4 motif (Fig. 3A), which forms a cap above the cAMP binding site. This region, including residues Arg61–Glu79 in KATms, contains a pair of proline residues (Pro52 and Pro78) uniquely present in KATms (Fig. 3A). We refer to this loop as the PP-loop. In KATms, the angle between the PP-loop and the C-terminal helix is broken at residues Pro80-Gly81 (the hinge at the end of the PP-loop described below), the linker peptide (residues Leu167–Leu178; Figs. 1A and 2), and residues Asp294–Gly296, suggesting high flexibility of these regions. This is in agreement with our earlier studies using hydrogen deuterium exchange followed by MS (8).

Some residues are shown in sticks (in the CNB domain: carbon, dark red; nitrogen, blue; oxygen, bright red; in AT domain: carbon, green; nitrogen, blue; oxygen, bright red; in the linker peptide: carbon, cyan; nitrogen, blue; oxygen, bright red). The missing part of the linker (linker gap) is marked. Acetyl-CoA from KATmt is shown in sticks (carbon, magenta; oxygen, red; nitrogen, blue; phosphorus, orange; sulfur, yellow). D, superposition of KATms and KATmt-cAMP (Protein Data Bank code 4AVB) on the CNB domain. KATms is shown in the same colors as in A, and KATmt_apo is shown in gray. Two highly conserved residues of KATms and the N and C termini are marked. Acetyl-CoA from KATmt is shown in sticks (carbon, light green; oxygen, red; nitrogen, blue; phosphorus, orange).

A

B

C

D

FIGURE 1. Crystal structure of KATms_WT. A, ribbon structure of KATms_WT (dark red, CNB domain; cyan, linker peptide; green, AT domain; orange, C-terminal helix). Some residues are shown in sticks (in the CNB domain: carbon, dark red; nitrogen, blue; oxygen, bright red; in AT domain: carbon, green; nitrogen, blue; oxygen, bright red; in the linker peptide: carbon, cyan; nitrogen, blue; oxygen, bright red). The missing part of the linker (linker gap) is marked. Top, schematic representation of the structure with the same color code as in ribbons. B, representation of the electron density in part of the KATms_WT structure (interface among the tip of the C-terminal helix, linker peptide, and CNB domain). KATms is shown in sticks with the same color code as in A. A 2Fo − Fc electron density map is shown as a gray mesh contoured at 1σ. Water molecules are marked as red spheres. C, superposition of KATms_WT and KATmt_apo (Protein Data Bank code 4AVA) on the CNB domain. KATms is shown in the same colors as in A, and KATmt_apo is shown in gray. Two highly conserved residues of KATms and the N and C termini are marked. Acetyl-CoA from KATmt is shown in sticks (carbon, magenta; oxygen, red; nitrogen, blue; phosphorus, orange; sulfur, yellow). D, superposition of KATms and KATmt-cAMP (Protein Data Bank code 4AVB) on the CNB domain. KATms is shown in the same colors as in A, and KATmt-cAMP is shown in gray. Two highly conserved residues of KATms and the N and C termini are marked. CAMP in KATmt-cAMP is shown in sticks (carbon, light green; oxygen, red; nitrogen, blue; phosphorus, orange).
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In KATmt, the β3-turn-β4 region equivalent to the PP-loop moves significantly inward upon cAMP binding and forms a caplike feature above the cAMP binding site (Figs. 1, C and D, and 3B). In KATms, the PP-loop is in an open position in the cAMP-free structure but may move inward and become fixed following cAMP binding. This hypothesis is indeed supported by results of previous hydrogen deuterium exchange followed by MS showing a significantly decreased deuterium exchange in this region of KATms in the presence of cAMP (8).

An important conserved residue in the CNB domain of KATms is Arg95, which was shown to contribute significantly to cAMP binding and following mutation abolished cAMP-mediated activation of acyltransferase activity (6). In KATmt-cAMP, the Nη1 atom of the corresponding Arg98 side chain is within hydrogen bonding distance with O2P of the phosphate moiety of the bound cAMP, but mutation of Arg98 to Ala did not abolish cAMP-mediated acetylation of USP (9). To elucidate the role of this residue, we determined the structure of the R95K mutant of KATms. Although the overall structure of KATms-R95K is very similar to KATms wild type, the mutation causes a conformational rearrangement at the cAMP binding site that now prevents access of cAMP. Although the CNB domains of KATms_WT and KATms-R95K superimpose closely with an r.m.s.d. of 0.6 Å, a significant deviation is seen in the PP-loop between KATms_WT and KATms-R95K (Fig. 3D) with the PP-loop of the R95K mutant moving slightly closer to the cAMP binding site. Moreover, we were surprised to find clearly defined electron density for showing that the final residues of the C-terminal helix of KATms_R95K take a different turn than that seen in KATms_WT, closing the cAMP binding site and making contacts with residues that at equivalent positions in KATmt-cAMP are involved in cAMP binding (Fig. 3D). For example, in the KATmt-cAMP structure, peptide nitrogens of Ala91 and Ser99 interact with the phosphate moiety of cAMP, and Glu89 interacts with the ribose ring (Table 2). However, in the KATms_R95K mutant, the carbonyl oxygen of Gln333 of the twisted C-terminal helix α10 makes hydrogen bonds to the peptide bond nitrogen as well as to Oy atom of Thr36 (equivalent to Ser99 in KATmt), and the Gln333 Nε2 atom forms hydrogen bonds to the peptide bond nitrogen of Ala88 (equivalent to Ala91), thus interfering with cAMP binding (Table 2). Additionally, the side chain of Gln333 in KATms_R95K forms a hydrogen bond to the amide nitrogen of Ile87, which is also situated in the cAMP binding site, as well as to the side chain of Glu86 whose equivalent in KATmt-cAMP, Glu89, forms a hydrogen bond with a ribose moiety of cAMP (Table 2). Therefore, it can be hypothesized that this strong engagement of the C-terminal residues with residues in the cAMP binding site in the KATms_R95K mutant contributes significantly to the low binding of cAMP as determined before for KATms_R95K (6). Thus, we suggest that the loss of interactions between the Arg side chain and the rest of the cAMP binding site upon mutation to Lys facilitates the inward turn and subsequent interactions of the C-terminal helix. In concert with this structural change, the PP-loop moves toward the center of the molecule, additionally closing the cAMP binding site albeit to a lesser extent than seen in KATms_WT (Fig. 3, C and D).

The Linker Peptide—The conserved fold of the CNB domain ends after helix α5 (Ile140) and is connected to the AT domain (starting at Asp235) with a 60-residue-long linker peptide (Fig. 2). The beginning of the linker peptide is well structured into the β8-strand-turn-β9-strand element, which after residue Val138 continues into a random coil structure defined in electron density up to residue Thr166. The electron density then breaks for 12 residues of sequence 167LNGPVEFSSETL178 and continues again with the residue Tyr179 at the beginning of a short semi-α6-helical fold, which then continues into helix α7 followed by the AT domain (Figs. 1A, 2, and 4A).

The general fold of the part of KATms linker peptide that is defined by the electron density matches relatively well with the equivalent region in KATmt_apo (Fig. 4A). However, superpo-
sition of the CNB domains of KATms and KATmt_apo reveals a structural deviation starting at the final turn of the α5 helix. This is followed by a prominent difference in the angle of the 8-strand-turn-9-strand motif between the two structures (Fig. 4). Although in KATms the linker residues Leu167–Leu178 are not seen, the corresponding residues in KATmt_apo are defined and make crucial contacts with the AT domain and acetyl-CoA (Fig. 4). Interestingly, residues 187SARVP191 in KATmt_apo are not defined (Fig. 2), whereas the corresponding region in KATms is defined and is exposed to solution.

The region of the linker peptide from Leu167 to Leu178 (KATms numbering) is relatively well conserved in mycobacteria (Fig. 4). Importantly, however, Pro170 in KATms seems to be unique because it is replaced with histidine in other mycobacterial KATs, including KATmt. In KATmt_apo, the respective His173 forms a stacking interaction of the imidazole side chain with the planar acetyl group of acetyl-CoA and a hydrogen bond between the imidazole Ne2 atom and carbonyl oxygen of Arg272 residing in the AT domain. These interactions enable the linker peptide to play an autoinhibitory role in KATmt_apo (Ref. 9 and Fig. 4f). Although upon cAMP binding to KATmt, His173 moves far out of the substrate binding site, enabling acetylation of the protein substrate (Fig. 4f).

The structure of KATms confirms the role of His173 in autoinhibition of KATms as well in binding of acetyl-CoA. The replacement of this His with a structurally distinct proline residue in KATms disables not only the interaction with potentially bound acetyl-CoA but may be also one of the reasons for very high structural flexibility of this region of the linker peptide in KATms. It is important to note that in KATmt acetyl-CoA is engaged by the linker peptide in either the absence (interaction with His173 in KATmt_apo) or presence of cAMP (interaction with the α6-helix in KATmt-cAMP). Therefore, the high flexibility of the linker and loss of the His side chain...
Glu234 to Ala in KATms reduced basal acyltransferase activity, to this conformation in KATms. Although Gln244 is conserved peptide discussed above, to preventing binding of acetyl-CoA which may contribute, along with the flexibility of the linker peptide (Fig. 4), located in the vicinity of the acetyl-CoA as well as the protein structure fits relatively well into the binding channel site in structures of KATms and consequently the absence of the autoinhibitory effect of the linker in KATms.

**AT Domain and Acetyl-CoA Binding Site**—The AT domain of KATms (residues Asp203—Val110) and KATmt apo (residues Asp206—Leu311) superimpose very well with an r.m.s.d. value of 1.1 Å. Deviations are seen in loops Glu211—Ile218 of KATms (KATmt Asp214—Val219) and Glu226—Ala230 (KATmt Asp227—Pro231). In both cases, the loops in KATms lack proline residues that are conserved in other mycobacteria (Pro218 in KATmt corresponds to Val217 in KATms, and Pro231 in KATmt corresponds to Ala230 in KATms), which may account for the different twists of these two loops.

There is an interesting detail in the acetyl-CoA binding site in KATms (Fig. 4). The acetyl-CoA taken from the KATmt apo structure fits relatively well into the binding channel site in KATms, indicating that the site is prebuilt in mycobacterial KATs. However, the binding channel in KATms is locked by the bridge formed by residues Glu244 and Ala278 (Fig. 4, B and E), which may contribute, along with the flexibility of the linker peptide discussed above, to preventing binding of acetyl-CoA to this conformation in KATms. Although Glu244 is conserved among other mycobacterial KATs, a Pro is found as the equivalent residue to Ala278 in other KATs (Fig. 4B). The presence of Ala278 instead of Pro may influence the shape and flexibility of the acetyl-CoA binding site in KATms.

The GNAT family of acyltransferases contains a conserved glutamate in the vicinity of the acetyl-CoA binding site (6). Catalysis in GNATs is believed to occur by direct nucleophilic attack on a substrate Lys residue by the conserved glutamate (23), potentially Glu234 in KATms and Glu235 in KATmt, located in the vicinity of the acetyl-CoA as well as the protein substrate binding site (Fig. 4, E and F). However, mutation of Glu234 to Ala in KATms reduced basal acyltransferase activity, which was increased in the presence of cAMP (6), clearly indicating that Glu234 was not the sole determinant for acyltransferase activity. We determined the structure of the E234A mutant protein and observed no dramatic change in the conformation of the acyltransferase domain. Indeed, in contrast to the KATms_R95K structure, the structure of KAT_E234A superposed almost completely with the KATms_WT structure with an overall r.m.s.d. of 0.4 Å (315 Ca atoms aligned). Therefore, we looked for an additional explanation that would account for the low catalytic activity of the E234A mutant protein in the absence of cAMP and that allowed recovery of catalytic activity on binding cAMP. The side chain of Asn269 is positioned close to the side chain of Glu234 (~3.5 Å) (Fig. 4D). Residues in other mycobacterial KATs corresponding to Asn269 are either Ala (KATmt) or Ser (Fig. 4B). Asn269 is positioned relatively far from the putative acetyl-CoA binding site, suggesting that following cAMP binding the conformational change that occurs may bring Asn269 closer to the active site, thereby assisting efficient catalysis.

**C-terminal Helix**—In general, the C-terminal α10-helix in KATms is less conserved in sequence in comparison with other mycobacterial KATs (8). In KATmt, the C-terminal helix was shown to play the role of a steric latch that contributes to the autoinhibition of KATmt in the absence of cAMP (9). Extending the length of this helix by a single amino acid results in considerable acyltransferase activity of KATmt even in the absence of cAMP. The amino acid alignment of mycobacterial KATs suggests that KATms is longer by one residue at the C terminus (6). Structural alignment of KATms_WT with KATmt (Fig. 2) revealed that the α10-helix in KATms is indeed longer by one residue at the C terminus than that of KATmt. This results in a different structure of the final twist of this helix and alters its structural relationship to the cAMP binding site and the PP-loop (Fig. 3 and Table 2). In contrast to the KATmt apo structure, the final residue of α10-helix, Glu333 in KATms_WT, does not protrude into the cAMP binding site (Fig. 3, A and C) but rather engages its side chain with hydrogen bond interactions to the residues Asp200, Tyr201, and Ala202 in the hinge between the linker peptide and AT domain (Table 2). This structural nuance may also contribute to the angular difference between the CNB and AT domains between KATms and KATmt and, thus, the higher affinity for cAMP binding to KATms than KATmt (6).

In KATmt apo, the cAMP binding site is closed by the protrusion of the tip of the C-terminal helix and the lid formed by the PP-loop, thus preventing access of cAMP. Therefore, this structure must breathe to capture cAMP when available. For KATmt, it was shown that to bind cAMP the C-terminal helix and the PP-loop have to be pulled apart with the PP-loop now shielding cAMP within the CNB domain (Fig. 3, B and E) and Ala202 in the hinge between the linker peptide and AT domain (Table 2). This structural nuance may also contribute to the angular difference between the CNB and AT domains between KATms and KATmt and, thus, the higher affinity for cAMP binding to KATms than KATmt.
Allosteric in cAMP-regulated Acyltransferases

structural changes that may occur in KATms following binding of cAMP and acetyl-CoA. We reasoned that mutation of Pro170 (analogous to His173 in KATmt) to a Lys residue would provide an acetylatable residue in the vicinity of the active site. Although KATms contains four Lys residues, none of them appeared to be acetylated in the protein as purified from *E. coli* (Fig. 5A). However, we observed that KATms P170K was robustly acetylated as purified, indicating the close positioning of Lys170 to the AT domain (Fig. 5B). Based on the conformational changes upon cAMP binding seen in KATmt (9), the linker peptide of KATms containing Lys170 would move away from the active site in the presence of cAMP and be more surface-exposed. To monitor this structural change, we tested the ability of the P170K mutant protein to be deacetylated by the sirtuin enzyme Rv1151c (7). We hypothesized that in the presence of cAMP the outward movement of acetylated Lys170 would allow greater access of Rv1151c and, therefore, more efficient deacetylation. As shown in Fig. 5A, right panel, KATms_Lys170 was marginally deacetylated in the absence of cAMP but was almost completely deacetylated by Rv1151c when bound to cAMP. Therefore, we can conclude that the conformational change that occurs in KATms on cAMP binding may indeed be similar to that seen in KATmt.

We then mutated Pro170 to a His residue that is found in KATmt, predicting that this mutation may now allow autoinhibitory interactions with the linker region in KATms. In agreement with our predictions, this single mutation resulted in almost complete loss of basal AT activity in KATms, whereas the addition of cAMP restored activity to levels seen in the wild-type protein (Table 3 and Fig. 5B). We also monitored cAMP binding affinity with the P170H protein and found that the IC50 showed an increased affinity for cAMP (KIC50 = 28.3 ± 24.5 versus 144.4 ± 10.9 nm for wild type; Fig. 5C), although the fraction of protein capable of binding cAMP was much lower.
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An important finding that emerged from our earlier mutational analysis as well as the structure of KATms was the putative role for Asn\(^{269}\) in the acetylation reaction in the presence of cAMP. To confirm this, we generated the N269A mutant protein and the double mutant E234A/N269A and compared their activities with the wild type and the E234A mutant. As we had reported earlier, the basal activity of the E234A mutant protein was low with significant activity seen in the presence of cAMP (6). The N269A protein retained high activity in the absence of cAMP (Table 3 and Fig. 6A) presumably because of the presence of Glu\(^{234}\) but showed only a marginal increase in activity in the presence of cAMP. The E234A/N269A double mutant protein showed low activity both in the absence and presence of cAMP.

To confirm that Asn\(^{269}\) assists in the enzymatic reaction in the presence of cAMP, we generated the R95K/E234A protein where binding of cAMP is prevented by the R95K mutation. This protein showed very low activity in both the absence and presence of cAMP (Table 3). KATmt contains an Ala at an equivalent position to Asn\(^{269}\). Therefore, we mutated Glu\(^{235}\) in KATmt to an Ala and monitored activity in either the presence or absence of cAMP. The mutant protein showed poor activity in either the presence or absence of cAMP (Fig. 6B), once again reiterating the importance of Asn\(^{269}\) in KATms.

Molecular Dynamics Simulation—Our results so far demonstrate that the structure of KATms has regions of high flexibility that account for its high basal acyltransferase activity. Therefore, we performed MD simulations on KATms WT and a model where Pro\(^{170}\) was mutated to His to identify regions of the molecule that contribute most to the dynamics of the

(Fig. 5C, inset). Therefore, this single residue change appeared to contribute significantly to altering the biochemical properties of KATms.

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TABLE 3
Activities of wild-type and mutant KATms proteins

| Protein       | cAMP     | +cAMP    |
|---------------|----------|----------|
| Wild type     | 19.9 ± 4.6| 27 ± 4.9 |
| P170H         | 1 ± 0.3  | 26.9 ± 5.1|
| E234A         | 2.8 ± 1.3| 22.2 ± 7.4|
| N269A         | 15 ± 6.8 | 17.7 ± 6.1|
| E234A/N269A   | 4.8 ± 2.9| 8.2 ± 4.5 |
| R95K/E234A    | 0.1 ± 0.1| 0.3 ± 0.01|

Continuous monitoring of enzymatic activity was performed as described in the text using equal amounts of individual proteins (500 ng). Values shown represent the mean ± S.D. of assays performed thrice.

![FIGURE 5. Critical role of Pro\(^{170}\) in KATms. A, purified wild-type KATms or mutant KATms_P170K was analyzed by Western blot analysis with acetyllysine antibody either directly or after incubation with cAMP and acetyl-CoA. KATms_P170K protein was acetylated as purified from E. coli, suggesting autoacetylation as a result of close proximity of Lys\(^{170}\) to the AT domain (left panel). Data shown are representative of assays performed twice. Incubation with the sirtuin enzyme of cAMP-free or cAMP-bound KATms_P170K resulted in efficient deacetylation only in the presence of cAMP (right panel), demonstrating that a conformational change had occurred in KATms following cAMP binding. Data shown are representative of assays performed twice. B, either wild-type KATms or the P170H mutant protein was incubated with USP and acetyl-CoA in either the absence or presence of cAMP, and acetylation of USP was monitored by Western blot analysis. C, either wild-type KATms or P170H mutant was incubated with \(\text{[^{3}H]cAMP}\) in the presence of varying concentrations of unlabeled cAMP as indicated. Data shown are the mean ± S.D. (error bars) of assays repeated thrice. Inset, binding assays using purified protein (1 µg for KATms WT (black bars) or 5 µg for KATms_P170H (white bars)) in the absence and presence of 1 mM unlabeled cAMP. Values shown are bound cpm of a representative experiment repeated thrice. ](image)
The PP-loop of the P170H mutant protein oscillated significantly during the simulation as seen from pronounced peaks for residues 66–76 (Fig. 7B). Moreover, the PP-loop of mutated KATms turned out to be more open relative to the KATms_WT during MD simulation as revealed by larger distances of the PP-loop opening (Fig. 7C). As a consequence, mutated KATms is thus expected to bind cAMP with even higher affinity than KATms_WT and therefore supports our experimental data (Fig. 5C). Simultaneously, the linker peptide seems to tend to close the protein substrate binding site, which is in agreement with lower acyltransferase activity in the absence of cAMP for the P170H mutant (Table 3 and Fig. 5B). Other parts of the structure stayed relatively stable during the MD simulations (Fig. 7B).

**DISCUSSION**

In this study, we identified critical differences in the structures of KATms and KATmt that now translate to dramatic differences in their biochemical properties. In contrast to KATmt, a significant fraction of KATms must exist in an active conformer, thus accounting for significant acyltransferase activity in the absence of cAMP. As we have shown here, single amino acid changes can have profound effects in determining the distribution of conformational ensembles of a protein.

KATms and KATmt share 60% identity in amino acid sequence and, therefore, similarity of the overall fold between their crystal structures. However, detailed inspection of the structural elements and spatial relation between them reveals (i) differences in the angle between the CNB and AT domains, (ii) a substantially more open access to the cAMP binding site in KATms, (iii) the absence of acetyl-CoA in the AT domain of KATms, and (iv) a longer C-terminal helix with a distinct final twist in KATms. The crucial structural elements that contribute to these differences are the PP-loop of the CNB domain, the linker peptide, and the C-terminal helix. Interestingly, sequence alignment of KATs from diverse mycobacterial species (6) shows that many residues that contribute to the unique properties of KATms are also unique to this protein.

The C-terminal helix attached to the AT domain is a unique structural element present in mycobacterial KATs. A salt bridge is formed between Asp<sup>73</sup> and Arg<sup>326</sup> from the C-terminal helix in KATmt, thereby closing access to the cAMP binding site. We suggest that this engagement of the C-terminal helix with the CNB domain partly contributes to the low affinity of KATmt for cAMP. In KATms, the C-terminal helix is one residue longer than that of KATmt, resulting in a dramatic change of the twist of the final turn of the helix that loosens its spatial interaction with the cAMP binding site. This lengthening of the helix results in the engagement of its tip residue, Gln<sup>333</sup>, with the body of the enzyme at the junction between the linker peptide and the AT domain. Thus, this contributes to further uncoupling of the already open conformation of the PP-loop, resulting in a distance between the corresponding residues Asp<sup>70</sup> and Arg<sup>325</sup> in KATms that is too large to allow the salt bridge formation seen in KATmt. Consequently, the KATms cAMP binding site is significantly less occluded than in KATmt, resulting in a 1000-fold higher affinity for cAMP in KATms (6).
Another striking difference between KATms and KATmt is the role of the highly conserved Arg in the cAMP binding site that anchors cAMP via interaction of its side chain with the phosphate group of the nucleotide (9). In KATms, Arg95 seems to be crucial for cAMP binding because the KATms_R95K mutant does not bind cAMP and shows no enhancement of acetylation in the presence of cAMP (6). This mutation also structurally blocks the cAMP binding site consequent to interaction with the C-terminal helix, resulting in a different angle between CNB and AT domains and the movement of the PP-loop closer to the nucleotide binding site than that seen in the wild-type structure. In contrast, mutation of the conserved Arg98 in KATmt to Lys or Ala did not completely abolish cAMP-regulated acyltransferase activity (Fig. 6B and Ref. 9, respectively). A plausible explanation could again be found in the shorter C-terminal helix in KATmt, which, because of its less flexible final turn, cannot lock the KATmt_R98K mutant at the interface with the CNB domain to the extent seen in KATms, thereby allowing a significant fraction of protein to adopt the cAMP-binding, active conformation.

The regulatory role of the linker peptide as seen in KATmt is reduced in KATms by the presence of Pro170 (His173 in KATmt), which contributes to high flexibility of the central part of the linker peptide of the KATms apo form. As a consequence, there is no occlusion of the protein substrate binding site in KATms as seen in KATmt via interaction of His173 with acetyl-CoA. Consequently, KATms can catalyze the transfer of the acyl group to the protein substrate in the absence of cAMP. However, the linker peptide of KATms must still contribute to the rotational dynamics of the two conserved domains that bring the enzyme into a conformation that allows cAMP binding and consequently increased AT activity. The conformational flexibility of the linker peptide noted in KATmt (9) was confirmed by biochemical analysis of KATms_P170K mutant (Fig. 5A).

Mutation of Pro170 to His abolished acyltransferase activity of KATms in the absence of cAMP, generating a cAMP-dependent protein acyltransferase as is KATmt. The presence of the imidazole side chain in KATms_P170H, therefore, may now help engage the linker region with Glu234 of KATms, thus pre-
venting substrate binding. However, it is interesting that although the P170H protein as purified contained a large fraction that was unable to bind cAMP the affinity of the fraction that retained cAMP binding was even higher that of the wild-type KATm. This can be explained by the presence of the longer C-terminal helix and more open PP-loop of KATms. Furthermore, the interaction of the linker peptide with the AT domain in KATms_P170H could result in an energetically more stable structure than that of the wild-type protein, thereby diminishing the fraction of protein present in an active conformation with open cAMP and protein substrate binding sites.

The relevance of the Pro170 in KATm was further supported by the results of molecular dynamics studies on KATms_WT and KATms_P170H, which showed prominent differences in PP-loop and substrate binding site flexibility between the two proteins. This confirms our hypothesis that Pro170 is one of the most crucial residues that enables KATms structural flexibility in the absence of cAMP, and the presence of a histidine residue as seen in KATmt allows activity of the enzyme only in the presence of cAMP.

Both available KATmt structures contain acetyl-CoA bound to the protein as purified from E. coli (9). In contrast, none of the KATms structures presented here have bound acetyl-CoA. The stacking interaction of His173 of the linker peptide with acetyl-CoA in KATmt could account for its continued presence in the AT domain even during purification procedures. Such an interaction is not possible in KATms due to the flexible linker and absence of the conserved histidine. Comparison of the acetyl-CoA binding site in KATms with that seen in KATmt and other GNAT superfamily members showed that although the acetyl-CoA binding site is basically prebuilt in KATms the entrance of the binding cleft is blocked by a bridge formed between residue Ala278 and Gln244. Thus, some motion will be required in the AT domain to allow acetyl-CoA binding during the enzymatic reaction. Recently solved structures of a GNAT member from Pseudomonas aeruginosa (24) in the absence or presence of acetyl-CoA reveal that a slight broadening of the cleft between the conserved loop (also called P-loop in GNATs (Fig. 2 and Ref. 25)) preceding α8-helix and the neighboring α9-helix (KATms annotation) in the AT domain occurs upon acetyl-CoA binding. Therefore, breathing of the KATms structure could pull apart the bridge between Gln244 of the P-loop and Ala278 of α9-helix and allow acetyl-CoA to slide into the binding cleft.

Another important difference between KATms and KATmt lies in the vicinity of the highly conserved Glu235 residue in KATmt (Glu234 in KATms). We show that mutation of Glu235 in KATmt abolished catalytic activity in the presence or absence of cAMP, whereas in KATms, the E234A mutant protein retained activity in the presence of cAMP. This indicates that Glu234 may not act as a general base during catalysis in KATms and may play a role in functional substrate binding. Because of structural similarity, a similar function could be proposed for Glu235 in KATmt. Indeed, His173 in the linker region of KATmt_apo probably mimics the position of Lys of the substrate (9), and it superimposes quite well with the substrate cis-4-hydroxy-L-proline seen in the structure of another GNAT acetyltransferase, Mpr1 (Protein Data Bank code 3W6X (26)).

Glu235 in KATmt_apo coordinates a water molecule, which in turn is hydrogen-bonded to His173 (Fig. 4E). This water remains at this position after cAMP binding (Fig. 4F) and, thus, is probably involved in coordination of the protein substrate. In Mpr1, Asn135 is found at an equivalent position to Glu235 in KATmt and was shown to be important for substrate recognition (26). Conversely, the residue critical for catalysis was found to be Asn178, which is located on the other side of the acetyl-CoA binding cleft. It was proposed that the side chain of Asn178 stabilizes the thiolate anion of acetyl-CoA after the degradation of the tetrahedral intermediate and that this thiolate anion could be protonated by the water molecule that is coordinated by the Asn178 side chain (26). KATmt and KATms both have asparagine at a position equivalent to Asn178 (Asn277 in KATmt and Asn276 in KATms) (Fig. 4, E and F). In fact, in the KATmt structure, Asn277 does offer an H-bond to acetyl-CoA oxygen OS5 and coordinates a water molecule and, thus, perhaps performs a role similar to that of Asn178 in Mpr1.

In contrast to KATmt, the E234A mutation in KATms was almost as active as the wild-type protein in the presence of cAMP. We show here that Asn269 is required to support the catalytic reaction in the absence of the side chain of Glu234 following the conformational rearrangement that occurs in cAMP-bound KATms. Asn269 in the wild-type protein might correctly orient and locate Glu234 in a manner similar to that seen in Mpr1 where Asn172 assists the function of Asn135 (present at a position equivalent to Glu234 in KATms) by maintaining its correct orientation and location (26). Thus, rather than in catalysis, both Glu234 and Asn269 in KATms may have a role to play in substrate positioning and perhaps substrate-induced conformational changes that are unique to KATms.

In conclusion, our structural analyses of wild-type and mutant KATms proteins reveal important differences that can account for the distinct biochemical properties of KATmt and KATms. Although our results clearly show that cAMP-free KATmt and KATms show unique structural features, we predict that the conformations of cAMP-bound structures are also likely to be subtly different. Clearly, these proteins have evolved to suit the distinct environments that M. tuberculosis and M. smegmatis inhabit and the demands placed on them to regulate their biology by cAMP and protein acylation.

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