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Acetylation by Eis and Deacetylation by Rv1151c of *Mycobacterium tuberculosis* HupB: Biochemical and Structural Insight

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Abstract

Bacterial nucleoid-associated proteins (NAPs) are critical to genome integrity and chromosome maintenance. Post-translational modifications of bacterial NAPs appear to function similarly to their better studied mammalian counterparts. The histone-like NAP HupB from *Mycobacterium tuberculosis* (*Mtb*) was previously observed to be acetylated by the acetyltransferase Eis, leading to genome reorganization. We report biochemical and structural aspects of acetylation of HupB by Eis. We also found that the SirT-family NAD⁺-dependent deacetylase Rv1151c from *Mtb* deacetylated HupB *in vitro* and characterized the deacetylation kinetics. We propose that activities of Eis and Rv1151c could regulate the acetylation status of HupB to remodel the mycobacterial chromosome in response to environmental changes.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01089. Figures showing comparison of the sequences of Hu proteins from various bacteria (Figure S1), the exact peptide fragments observed by mass spectrometry (Figure S2), and comparison of crystal structures of acetyltransferases in complex with peptides (Figure S3). A table of X-ray diffraction data collection and structure refinement statistics for the Eis-CoA-HupB_ *Mtb* 9-mer complex (Table S1) (PDF)

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Author Contributions

S.G.T. and O.V.T. designed the project. K.D.G. performed all biochemical experiments. T.P., A.H.P., and O.V.T. performed the structural biology work. M.J., M.S.R., O.S., J.S., and J.E.P. conducted the mass spectrometry experiments. K.D.G., O.V.T., and S.G.T. wrote the manuscript, and all other authors provided comments. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Graphical abstract

Acetylation of proteins is a co- or post-translational modification with important regulatory functions in eukaryotic metabolism, including gene expression.^{1,2} Histone acetylation by histone acetyltransferases (HATs) and the reverse deacetylation by histone deacetylases (HDACs) are especially significant due to the roles of these enzymes in regulation of gene expression through chromatin remodeling and other changes in biomolecular interactions.^{3,4} HATs and HDACs are attractive targets of therapeutically promising inhibitors.^{5–7}

Despite much progress in understanding protein acetylation in eukaryotes, similar post-translational modifications in prokaryotes are much less studied. Over 100 different lysine residues in 85 *Escherichia coli* proteins are known acetylation sites.⁸ Changes in protein acetylation were found to reprogram carbon source utilization pathways in *Salmonella enterica*.⁹ These modifications may be responsible for transitions of the bacterium between different growth phases. Acetylation may mediate bacterial adaptation and virulence, particularly in pathogens such as *Mycobacterium tuberculosis* (*Mtb*), which is known to survive in the hostile environment of the macrophage.^{10,11} Tuberculosis (TB) causes millions of deaths each year and poses serious challenges due to the large number of individuals worldwide with latent TB (an asymptomatic, noncontagious infection), HIV/AIDS-associated TB, multi-drug-resistant (MDR) TB, and extensively drug-resistant (XDR) TB. Understanding post-translational protein modifications in this pathogenic bacterium that likely regulate its transitions between replicating and nonreplicating states would not only provide deeper insight into the regulation of bacterial metabolism, but also suggest new potential drug targets.

The enhanced intracellular survival (Eis) protein is a unique GNAT-family bacterial acetyltransferase.¹² Upregulation of Eis due to mutations in its promoter has been shown to cause clinically important resistance of *Mtb* to the aminoglycoside kanamycin (KAN).¹³ The resistance arises due to aminoglycoside acetylation by Eis, resulting in an inactive acetylated product. In addition to the mycobacterial cytoplasm, Eis has been found outside of the bacterial cell in the host macrophage and in macrophage culture supernatant, where it can acetylate human histone H3¹⁴ and DUSP16,¹⁵ and modulate the secretion of IL-10 and TNF α by human monocytes.¹⁶ These acetylation functions of Eis were implicated in several host cell processes: T-cell function,¹⁷ negative modulation of autophagy, inflammation, and cell death via inhibition of production of reactive oxygen species.¹⁸

Recently, Eis from *Mtb* was demonstrated to acetylate HupB (Rv2986c), a nucleoid-associated protein (NAP) in *Mtb*, which is homologous to a ubiquitous bacterial histone-like protein Hu, both *in vitro* and in the mycobacterial cell.¹⁹ HupB and Hu contain a DNA binding histone-like core, but unlike Hu, HupB contains an additional C-terminal histone tail-like lysine-rich region.¹⁹ HupB expression has been shown to be upregulated during infection, suggesting that it plays a role in *Mtb* virulence.²⁰ HupB was demonstrated to organize the *Mtb* chromosome, in concert with topoisomerase I.²¹ Acetylation of the basic C-terminal region of HupB by Eis weakens DNA binding to HupB *in vitro* and causes chromosome reorganization.¹⁹ In addition to its function in chromosome remodeling, HupB regulates production of siderophores in *Mtb*²² and is itself regulated by iron.²³

The mechanism regulating the acetylation of HupB in the mycobacterial cell is not known, in part, because the enzyme that can deacetylate HupB is yet to be identified. The genome of *Mtb* encodes a homologue of eukaryotic silent information regulator 2 (Sir2; Rv1151c). This protein was demonstrated to deacetylate an acetylated lysine of large acetyl-CoA synthetase (ACL),²⁴ an *Mtb* enzyme that is regulated by post-translational acetylation.^{25,26} In *Mycobacterium smegmatis* (*Msm*), the Sir2 homologue (DAc1) deacetylates fatty acyl-AMP ligase FadD33 involved in siderophore production, reversing its inhibition by acetylation.²⁷ Sir2 homologues in archaea and other bacteria have also been shown to modulate enzyme activity and transcription.^{28,29}

To gain mechanistic insight into acetylation of HupB by Eis and to investigate whether deacetylation can be carried out by Rv1151c, we performed biochemical studies of HupB acetylation by Eis and its deacetylation by Rv1151c as well as structural studies of Eis in complex with a lysine-rich HupB peptide.

MATERIALS AND METHODS

Materials and Instrumentation

Reagents for cloning were obtained from Invitrogen (e.g., chemically competent *E. coli* TOP10, BL21 (DE3), and BL21 (DE3) pLysS strains), NEB (e.g., restriction enzymes, T4 DNA ligase, and Phusion DNA polymerase), Novagen (e.g., pET28a and pET17b vectors), and Sigma-Aldrich or Integrated DNA Technologies (e.g., primers for PCR). The genomic DNA from *Mtb* H37Rv utilized for PCR was obtained from BEI resources. DNA sequencing was performed at the Advanced Genetic Technologies Center at the University of Kentucky. Bacteria were lysed using a Q500 Qsonica sonicator. Protein concentrators and membrane filters were obtained from Millipore. DTNB, AcCoA, H1_ *Bta* (cat. # H5505), and standard reagents for protein purification and biochemical assays were obtained from Sigma-Aldrich and used without further purification. The heparin-agarose column (cat. # 17-0407-01) was obtained from VWR. [³H]AcCoA was purchased from PerkinElmer. The peptides (HupB_ *Mtb* 9-mer and 29-mer) were purchased from AnaSpec. All spectrophotometric assays were performed using a SpectraMax M5 multimode microplate reader from Molecular Devices. A Packard TRI-CARB 2900TR liquid scintillation analyzer (PerkinElmer) was used to measure tritium decay. Nano-LC ESI-TOF MS/MS analysis was performed using the maXis ESI-Q-TOF (Bruker Daltonics) mass spectrometer online with the Dionex model nanoRS nanobore HPLC. The desalting trap column used was 0.3 × 5

mm, 5 μ m, C18 PepMap, 120 Å (Dionex, San Jose, CA), and the analytical column used was C18 PepMap, 0.075 $\times$ 250 mm, 2 μ m, 120 Å (Dionex). X-ray diffraction data were collected at the LS-CAT beamline 21-ID of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL, USA).

Cloning, Expression, and Purification of HupB_*Mtb*

The *hupB* gene was amplified by PCR from the genomic DNA of *Mtb* H37Rv by using the forward and reverse primers 5'-GGGCCCCATATGAACAAAGCAGAGCTC-3' and 5'-GCAGCCGGATCCCTATTTTCGCACCCCG-3' with the *Nde*I and *Bam*HI restriction sites (underlined), respectively. The *hupB* gene was ligated into pET17b linearized with *Nde*I and *Bam*HI and transformed into chemically competent *E. coli* TOP10 cells by using standard cloning protocols to afford the pHupB-pET17b plasmid. For large-scale protein production and purification, the pHupB-pET17b plasmid was transformed into chemically competent *E. coli* BL21 (DE3) pLysS cells. Briefly, 1 L of Luria-Bertani (LB) medium was inoculated with 10 mL of an overnight culture. The cells were grown at 37 °C (200 rpm) to an OD₆₀₀ nm of 0.5, and protein expression was induced with 0.5 mM IPTG (final concentration). The culture was cooled to 20 °C and kept growing at 200 rpm. After 16 h, the bacteria were collected and washed with STE buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM EDTA). The cell pellet was resuspended in Buffer A (10 mM Tris-HCl pH 7.5, 4 mM EDTA, 150 mM NaCl, 5 mM DTT, and 5% (v/v) glycerol). The suspension was frozen at -80 °C for at least 20 h before lysis by sonication on ice (70% duty cycles, four cycles of alternating 2 s “on” and 10 s “off” over a period of 2 min for each cycle) of the thawed mixture. The lysed cell pellet was clarified by centrifugation at 16 000 rpm, 4 °C, for 45 min. The lysate was filtered through a 45 μ m low-protein-binding PVDF membrane filter and loaded onto a heparin-agarose column equilibrated with Buffer A. A stepwise gradient was used to elute the HupB_*Mtb* protein from the heparin column using 15 mL fractions of Buffer A containing 300, 450, 600, 750, 900, 1000, and 2000 mM NaCl. Fractions containing pure HupB_*Mtb*, as determined by SDS-PAGE, were pooled and concentrated using a 10000-MWCO Amicon centrifugation device. The protein was kept at 4 °C and was stable for 2 weeks. A total of 1.8 mg of HupB_*Mtb* was obtained per L of culture. Concentration of HupB_*Mtb* was determined by the Bradford protein assay.

Determination of Kinetic Parameters for a 29-mer C-Terminus HupB_*Mtb* peptide, Full-Length HupB_*Mtb*, and H1_*Bta* by UV-vis Assay

All kinetic parameters were determined by using a UV-vis assay monitoring the reaction of Ellman's reagent (DTNB) with the CoA released during the acetylation reaction at 412 nm ($\epsilon = 14150 \text{ M}^{-1}\text{cm}^{-1}$) at 25 °C. 100- μ L reactions were initiated by the addition of a mixture of AcCoA and DTNB. All measurements were taken every 30 s for 1 h. All experiments were performed in triplicate. Detailed conditions for each substrate are as follows.

For the 29-mer C-Terminus HupB_*Mtb* Peptide—The 29-mer TKAPAKKAAAKRPATKAPAKKATARRGRK (0, 1, 5, 10, 25, 50, 100, or 250 μ M) was incubated with Eis (0.25 μ M), AcCoA (250 μ M), and DTNB (2 mM) in Tris-HCl (50 mM, pH 8.0). The first 5 min of the reactions were used to calculate the initial rates.

For the HupB_Mtb Protein—HupB_Mtb (0.25, 0.5, 1, 2.5, 5, 10, and 25 μM) was incubated with Eis (0.25 μM), AcCoA (1 mM), and DTNB (2 mM) in Tris-HCl (50 mM, pH 8.0). The entire reaction time (1 h) was used to determine the initial rates.

For the H1_Bta Protein—The H1_Bta from *Bta* (0, 10, 25, 50, 100, or 250 mg/mL) were incubated with Eis (0.25 μM), AcCoA (1 mM), and DTNB (2 mM) in Tris-HCl (50 mM, pH 8.0). The entire reaction time (1 h) was used to calculate the initial rates.

Acetylation of H1_Bta by Eis Homologues

The acetylation of H1_Bta was monitored by using the same UV-vis assay as for the kinetic experiments. Briefly, reactions (200 μL) containing H1_Bta (100 $\mu\text{g/mL}$), Eis (0.5 μM), AcCoA (500 μM), and DTNB (2 mM) in Tris-HCl (50 mM, pH 8.0) were monitored for 1 h taking measurements every 30 s. The data were plotted as a function of absorbance and time to show the reactivity of each Eis homologue with H1_Bta.

Acetylation of HupB by Other AACs

The ability of other AACs to acetylate HupB was examined radiometrically using a series of seven mono- and bifunctional AACs (AAC(2')-Ic, AAC(3)-IV, AAC(3)-Ib, AAC(6')-Ib', AAC(6')-IId, AAC(6')-Ie, and AAC(3)-Ib/AAC(6')-Ib') and Eis Y126A, a known inactive Eis mutant¹² with [³H]AcCoA. Briefly, AAC enzyme (0.5 μM) was incubated with HupB_Mtb (2.5 μM), AcCoA (100 μM), and [³H]AcCoA (~142 000 DPM per reaction) in Tris-HCl (50 mM, pH 8.0) for 10 min. Reactions (25 μL) were initiated by the addition of HupB_Mtb. In 10 min, the reactions were quenched by addition of 10% trichloroacetic acid (TCA; 100 μL). After being washed with another 100 μL of TCA, the reactions were resuspended in formic acid (100 μL), added to scintillation liquid, and quantified. All AAC enzymes were purified as previously described^{12,30,31} and tested at their optimal pHs (pH 6.6 for AAC(6')-Ie, AAC(3)-IV, AAC(3)-Ib, and AAC(6')-Ib', and pH 7.0 for AAC(2')-Ic) and at pH 8.0. Experiments were normalized to Eis wt.

Identification by Mass Spectrometry of Lysine Residues of HupB_Mtb and H1_Bta Acetylated by Eis

Enzymatic reactions (100 μL) for mass analysis were incubated at room temperature in microcentrifuge tubes overnight and contained HupB_Mtb or H1_Bta (~5 μM), Eis (0.5 μM), and AcCoA (500 μM) in Tris (50 mM, pH 8.0). For the mass spectrometry experiments, samples were separated using SDS-PAGE-gel (Invitrogen NuPAGE 4–12%, MES running buffer) and stained with Imperial Protein Stain (Pierce). Stained bands were excised and digested with Trypsin (Promega) overnight. Tryptic peptides were extracted, concentrated, and submitted for nano-LC-MS/MS analysis. A gradient of 2–55% B in 90 min was applied using 0.1% formic acid in H₂O (solvent A) and 80% acetonitrile/0.1% formic acid in H₂O (solvent B). The Bruker Daltonics online nanospray source was operated at 1200 V with a drying gas of nitrogen flow at 6 L/min. The capillary temperature was set to 150 °C and the instrument acquired line spectra of *m/z* 50 to 2,200. The data were analyzed and searched against multiple FASTA databases using the Bruker ProteinScape program version 2.1 and Mascot program version 2.4. The data were first searched against the NCBI database followed by a search against the Uniprot/Swissprot database using an expanded set

of variable modification. A third search was done using a combination of user supplied sequences and known/expected contaminant sequences. The data were also processed by the PEAKS software against the known sequences using PEAKS homology searching and post-translational modifications (PTM) search.

Inhibition of HupB_*Mtb* Acetylation

Three previously reported Eis inhibitors (**1**–**3**)^{32–35} were tested for their ability to inhibit the acetylation of the HupB_*Mtb* 29-mer peptide. Reactions containing Eis (0.25 μ M), HupB_*Mtb* 29-mer (50 μ M), AcCoA (500 μ M), DTNB (2 mM), and inhibitor (**1** = 3.1 μ M, **2** = 0.5 μ M, **3** = 1.8 μ M) in Tris-HCl (50 mM, pH 8.0) were performed in triplicate. The first 5 min of reaction were used to calculate the initial rates and normalized to the reaction containing no inhibitor. We also carried out a dose–response assay with compound **2** at four concentrations (2, 22, 100, and 200 μ M) using the same protocol.

Cloning, Expression, and Purification of Rv1151c Protein

The Sir2-like NAD-dependent deacetylase (*Rv1151c*) was PCR amplified from the genomic DNA of *Mtb* by using the forward and reverse primers 5' - TATGTTCATATGCGAGTGGCGGTGCTC-3' and 5' - GCCCATCTCGAGCTATTCAGCAGGGC-3' with the *Nde*I and *Xho*I restrictions sites (underlined), respectively. The *Rv1151c* gene was ligated into the pET28a linearized with *Nde*I and *Xho*I and transformed into chemically competent *E. coli* TOP10 cells by using standard cloning protocols to afford the pRv1151c-pET28a plasmid. For large-scale protein production and purification, the pRv1151c-pET28a plasmid was transformed into chemically competent *E. coli* BL21 (DE3) cells. The deacetylase was expressed similarly to a previously reported preparation.²⁴ Briefly, 10 mL of an overnight culture was added to 1 L of LB medium. The culture was grown to an OD₆₀₀ nm of 0.6, and the protein production was induced with 0.5 mM IPTG. The cells were then grown overnight at 25 °C. The cell pellet was lysed in sodium phosphate (50 mM, pH 8.0) with NaCl (200 mM) and glycerol (10% v/v), and purified by using a protocol previously reported for Eis purification.¹² The fractions containing pure Rv1151c were pooled and dialyzed in sodium phosphate (50 mM, pH 8.0) before being concentrated by using a 10000-MWCO Amicon centrifugation device. The protein was flash-frozen in liquid nitrogen and kept at –80 °C. Purification yielded 1.0 mg of Rv1151c per L of culture.

Determination of Kinetic Parameters for the Deacetylation of AcHupB_*Mtb* by Rv1151c

The deacetylation kinetic parameters of the Sir2-like Rv1151c_*Mtb* protein were determined radiometrically. Here, HupB_*Mtb* (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 25 μ M) was first acetylated in 12.5 μ L (50 mM Tris-HCl, pH 8.0, 30 mM NaCl) reactions by incubation at room temperature with Eis (0.5 μ M), AcCoA (100 μ M), and [³H]AcCoA (~227,000 DPM per reaction). These reactions were incubated overnight. The next day, a solution (12.5 μ L) containing the deacetylase Rv1151c_*Mtb* (0.1 μ M), NAD⁺ (2 mM), Tris-HCl (50 mM, pH 8.0), NaCl (137 mM), and KCl (40 mM) was added to initiate the deacetylation reaction (all concentrations are final concentrations). In 10 min, the reactions were quenched with 10% TCA precipitating the protein, which was then pelleted by centrifugation. The pellet was

resuspended in 10% TCA and pelleted again. For quantification by scintillation counting, the protein pellet was resuspended in formic acid and added to the scintillation liquid. The experiments were performed in triplicate. It is important to note that the added salts needed for the deacetylation reactions quenched the Eis acetylation activity. This was confirmed by the UV-vis assay at 33 mM and 150 mM NaCl.

Crystallization and Crystal Structure Determination of Eis-HupB_*Mtb* Peptide Complex

Crystals of Eis-CoA-HupB_*Mtb* peptide complex were grown and cryoprotected following the previously reported crystallization protocol, where we used wild-type Eis and 1 mM HupB peptide instead of tobramycin.³⁶ The X-ray diffraction data were collected at 100 K at beamline 21-ID of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL). HKL2000³⁷ was used to index, integrate and scale the data. The crystals were highly sensitive to radiation damage. This factor, combined with the low symmetry and non-isomorphism among crystals resulted in somewhat incomplete data (Table S1). The lack of completeness was counterbalanced by the high model quality and the use of a 3-fold noncrystallographic symmetry in initial refinement cycles, ultimately yielding a high-quality electron density map. The structure was determined by molecular replacement with one protomer of Eis from our prior high-resolution crystal structure (PDB ID: 3R1K¹²) used sequentially as the search model in PHASER.³⁸ Refinement and model building were performed iteratively by using REFMAC5³⁹ and COOT.⁴⁰ A strong difference $F_o - F_c$ electron density was identified that accounted for a part of the CoA molecule in each of the three CoA binding sites of the three Eis protomers in the asymmetric unit as well as the C-terminal part of the HupB_*Mtb* 9-mer peptide (AKK) in the substrate binding site of two out of three protomers. Further analysis by generating more rigorous, polder omit $mF_o - DF_c$ electron density maps⁴¹ in the Eis substrate binding sites of different protomers allowed us to build an additional Pro residue (to yield PAKK region) in one of the sites. A part of the phosphopantetheinyl arm of CoA was not observed likely to its disorder, consistent with similar disorder observed in crystal structures of Eis in complexes with inhibitors.^{32,42} The data collection and refinement statistics are summarized in Table S1. The coordinates and structure factors for the Eis-CoA in complex with the *Mtb* HupB (HupB_*Mtb*) peptide were deposited in the Protein Data Bank with PDB accession number 6B0U.

RESULTS

Sequence Analysis of HupB and Its Homologues

We define HupB as a protein with an Hu-like N-terminal region and a lysine-rich C-terminal region (Figure S1). Because Eis homologues are found in several diverse bacteria^{43–46} and Eis from *Mtb* acetylates HupB,¹⁹ Eis may have coevolved with HupB to perform a similar function in other bacteria. A BLAST search using HupB_*Mtb* yielded homologues in many *Mycobacteria* and other genetically related actinomycetes, which also contained an Eis homologue. More genetically distant bacteria, such as *Bacillus anthracis*,⁴⁵ which contain more distant Eis homologues, do not appear to contain a HupB homologue (but contain Hu). The co-occurrence of Eis and HupB in actinomycetes is very strong. For example, another mycobacterial pathogen, *Mycobacterium leprae*, lacks both Eis and HupB homologues. *Mycobacterium abscessus* (*Mab*), *Msm*, and *Tsukamurella paurometabola* (*Tpa*) all encode

Eis and HupB homologues (Figure S1). HupB from *Mab* and *Msm* are 70–80% identical in sequence to HupB_*Mtb* and contain one additional lysine in the C-terminal tail compared to HupB_*Mtb*. HupB from *Tpa* is 64% identical to its *Mtb* counterpart and contains 11 more lysine residues than HupB_*Mtb*.

Full-length HupB and mammalian histone H1 are ~35% identical in sequence. For example, in three *Bos taurus* histone H1 isoforms (H1.1, H1.2, and H1.3), out of 40 residues that are identical in all four proteins, 20 are lysine residues that reside in the lysine-rich C-terminal region (Figures 1 and S2).

Kinetics of Acetylation of Histone Proteins and Peptides by Eis

The steady-state kinetic parameters of Eis-catalyzed acetylation were determined by an established UV–vis assay using three substrates: (1) a mammalian histone H1 from *Bos taurus* (H1_*Bta*) (Figure 2A), (2) a 29-mer peptide corresponding to residues 186–214 of the HupB_*Mtb* C-terminal lysine-rich tail (Figure 2B), and (3) full-length HupB_*Mtb* (Figure 2C). The Michaelis–Menten kinetic parameter values for acetylation of H1_*Bta* were $K_m = 4.9 \pm 1.1 \mu\text{M}$ (on a total protein concentration basis) and $k_{\text{cat}} = 0.72 \pm 0.05 \text{ min}^{-1}$, yielding the catalytic efficiency of $2450 \pm 580 \text{ M}^{-1} \text{ s}^{-1}$. We then determined the steady-state kinetic parameters for the acetylation of the 29-mer peptide. These acetylation kinetics were $K_m = 52 \pm 12 \mu\text{M}$ and $k_{\text{cat}} = 29 \pm 2 \text{ min}^{-1}$, yielding the catalytic efficiency of $9300 \pm 2200 \text{ M}^{-1} \text{ s}^{-1}$. The K_m and k_{cat} values for the acetylation of full-length HupB_*Mtb* were $1.0 \pm 0.2 \mu\text{M}$ and $0.33 \pm 0.02 \text{ min}^{-1}$, respectively, yielding the catalytic efficiency of $5500 \pm 1150 \text{ M}^{-1} \text{ s}^{-1}$.

We also observed that, similarly to Eis from *Mtb*, the Eis homologues from *Msm*, *Mab*, and *Tpa* can acetylate H1_*Bta* (Figure 3). The acetylation of H1 progressed at rates of 0.028, 0.058, 0.118, and $0.160 \mu\text{M min}^{-1}$ for Eis from *Msm*, *Mtb*, *Mab*, and *Tpa*, respectively.

We tested seven other AAC enzymes to understand if the Eis catalyzed acetylation of HupB is a function of any aminoglycoside acetyltransferase or specifically Eis. Eis Y126A was also tested as an inactive control. The AACs were tested at their optimum pH for modifying aminoglycosides and at pH 8.0, the optimum activity of Eis. No other AACs were able to acetylate HupB at either of the pHs tested (Figure S4).

Identification of HupB_*Mtb* and H1_*Bta* Residues Acetylated by Eis

We carried out an *in vitro* HupB_*Mtb* acetylation assay with Eis and analyzed the acetylation sites by nano-LS-MS/MS. We identified 31 acetylated Lys residues, which were located not only in the C-terminal lysine-rich region, but also in the core region (Figure 1). In addition to the previously reported acetylation sites (pale green ovals in Figure 1),¹⁹ we identified five additional Eis-catalyzed acetylation sites: Lys72, Lys113, Lys122, Lys183, and Lys187 (green rectangles in Figure 1). Having demonstrated that our method was highly sensitive, we determined which residues of H1_*Bta* were acetylated by Eis. The H1_*Bta* is a mixture of three isoforms: H1.1, H1.2, and H1.3. The sites acetylated by Eis in the presence of AcCoA on the three H1_*Bta* isoforms were also determined analogously. All three H1_*Bta* isoforms were found to be acetylated by Eis: H1.1 (yellow rectangles, 5 Lys

residues, 17% Lys coverage), H1.2 (red rectangles, 28 Lys residues, 52% Lys coverage), and H1.3 (blue rectangles, 11 Lys residues, 24% Lys coverage).

There are 22 conserved lysines in the HupB_*Mtb*-H1_*Bta* alignment, including one residue where H1.1_*Bta* contains an arginine instead of lysine (residue 166 in HupB_*Mtb* numbering) (Figure S1). On the basis of our data, five of these residues (117, 129, 183, 201, and 206, HupB_*Mtb* numbering) are acetylated only in HupB_*Mtb*, 12 (72, 113, 116, 146, 147, 166, 170, 174, 178, 187, 191 and 196, HupB_*Mtb* numbering) on lysines found in HupB_*Mtb*, and at least one isoform of H1_*Bta* including the mismatched arginine from H1.1_*Bta*, and 5 (155, 156, 159, 167, and 214, HupB_*Mtb* numbering) that were only found in H1_*Bta* (Figure 1).

Inhibition of HupB_*Mtb* Acetylation

We recently reported discovery and development of potent small molecule Eis inhibitors of several different chemical scaffolds.^{32–35,42} Three molecules of unrelated scaffolds (Figure 4A) that we reported as inhibitors of Eis-catalyzed acetylation of KAN^{32–35} were tested for inhibition of acetylation of the HupB_*Mtb* 29-mer peptide by Eis (Figure 4B). To ensure a standard comparison between the inhibitors, we used them at concentrations in the sub- to low-micromolar range that correlated with their IC₅₀ values for their inhibition of KAN acetylation by Eis.^{32–35} All three scaffolds reduced the Eis activity to 20–30% (Figure 4B), indicating their potent inhibition of Eis acetylation of HupB. In addition, we carried out a dose–response assay with compound **2** (Figure 4C). The acetylation activity was found to be 10%, 10%, 12%, and 61% as active as the reaction with no inhibitor when in the presence of 200, 100, 22, and 2 μ M of compound **2**, respectively.

Deacetylation of Acetylated HupB_*Mtb* by Rv1151c

The deacetylating activity of recombinant Rv1151c was measured by using a previously described fluorometric assay.²⁴ To determine the ability of Rv1151c to deacetylate HupB_*Mtb* acetylated by Eis (AcHupB), a radiometric assay was used to first label HupB with [³H]Ac-groups using Eis. The [³H]AcHupB was then tested as a substrate of Rv1151c and the steady-state kinetic parameter values for this catalytic reaction were $K_m = 4.7 \pm 0.6$ μ M, $k_{cat} = 1.27 \pm 0.06$ min^{−1}, yielding the catalytic efficiency of 4500 ± 600 M^{−1} s^{−1} (Figure 5A). The deacetylation assay conditions (specifically, 133 mM NaCl) did not support acetylation of HupB by Eis (Figure 5B); therefore, inactivation of Eis, which was still present in the reaction mixture, was not necessary.

Crystal Structure of Eis in Complex with a HupB_*Mtb* peptide

We obtained crystals of Eis_*Mtb* in complex with CoA and a basic 9-mer peptide, ATKAPAKKA, whose sequence is repeated four times in the HupB_*Mtb* sequence (residues 140–148, 149–157, 185–193, and 199–207). The Lys residues in these regions can be acetylated by Eis (Figure 1). We determined a crystal structure of this complex at the resolution of 2.80 Å (Figure 6A,B and Table S1). The crystals contain three monomers of Eis per asymmetric unit, with the reported biological hexamer assembly¹² generated by crystal symmetry operations. Two out of three Eis monomers contained a strong continuous omit F_0 – F_c electron density in the substrate-binding pocket of the enzyme, consistent with

the AKK peptide region. The polder omit map analysis⁴¹ allowed us to extend the modeled AKK peptide to PAKK in one of the binding pockets (Figure 6A,B). The site occupied by this peptide was demonstrated to be occupied by tobramycin (Figure 6C)³⁶ and Eis inhibitors (e.g., Figure 6D) by our previous structural studies.^{32,34,35,42} The electron density that could account for the rest of the peptide was not found, apparently due to the dynamic nature of that region. The side chain amino group of Lys8 (underlined in ATKAPAKKA) appears to be positioned for N_ϵ -acetylation by Eis, occupying the site where the amino groups of tobramycin³⁶ and the acetamide group¹² were observed in our previous crystal structures. Acetylation of a Lys in this sequence context by Eis was observed for Lys147, Lys192, and Lys206 of HupB_*Mtb* (Figure 1). The aliphatic parts of the side chain and the main chain of this residue are flanked on one side by Phe24. The side chain amino group of this Lys is in the electronegative environment of the C-terminal carboxyl group of Eis and at a hydrogen bonding distance to the carbonyl oxygen of His119. The aliphatic stem of the preceding Lys7 residue is located in a cleft lined by hydrophobic residues Phe84, Ile28, and Trp36, Met65, Leu63, and Ala33. The N_ϵ amino group is located in a large void; this group could be solvated and interacting with protein through water molecules that are not resolved in the electron density map. The nonpolar portion of Ala6 interacts with the aliphatic part of the side chain of Glu401 and is solvent exposed on the other side. The flexible portion of the peptide is extended into this solvent space.

DISCUSSION

Eis is an enzyme whose upregulation is observed in approximately one-third of KAN-resistant clinical isolates of *Mtb*.¹³ Eis can acetylate a variety of aminoglycosides, most of them at multiple amino group positions.¹² Eis contains a large and complex substrate binding cavity formed by juxtaposition of two GNAT folds and contribution of a C-terminal region extended from a sterol carrier protein fold.¹² Therefore, it is not a surprise that Eis can acetylate aminoglycosides and peptides. We previously demonstrated that in addition to aminoglycosides, Eis could acetylate a neurogranin peptide and the nonribosomal peptide antibiotic, capreomycin.⁴⁷ In the same study, we found that a tetralysine (KKKK) peptide was a substrate for Eis, whereas L-Lys, dilysine (KK), and trilycine (KKK) were not substrates for this acetyltransferase. Here, we demonstrate that Eis can extensively acetylate a large number of Lys residues in the histone-like protein HupB from *M. tuberculosis*. The acetylation sites lie both in the core and the tail region. Even though the extent of acetylation *in vitro* is more extensive than that observed for HupB purified from mycobacteria,¹⁹ the latter also includes both the core and the tail. Additional acetylation sites may be accessible *in vitro* due to the absence of other HupB interacting partners or because of the absence of deacetylase activity.

Differences in the apparent kinetics of acetylation of various substrates by Eis (Figure 2) are, at least partially, explained by the different numbers of acetylation sites and the intrinsic efficiency of their acetylation. K_m value for HupB_*Mtb* is 50- and 4.6-fold lower than for the 29-mer peptide and H1_*Bta*, respectively, and over 1000-fold lower than the previously reported value for the tetralysine ($K_m = 1130 \pm 168 \mu\text{M}$ ⁴⁷). These differences arise from a much larger number of sites on full-length HupB_*Mtb* and H1_*Bta* compared to the peptide substrates (i.e., if expressed in terms of the concentrations of acetylation sites, the K_m values

would be more similar for all substrates). Eis likely binds the tetralysine peptide less strongly than the Ala-containing motifs. The K_m value for HupB that we determined ($1.0 \pm 0.2 \mu\text{M}$) is comparable in magnitude to previously published data ($2.4 \pm 0.4 \mu\text{M}$),¹⁹ with only a ~2-fold difference. The maximum turnover rates of acetylation of different substrates are ranked in the same order as the respective K_m values: H1_*Bta* had a 2.2-fold faster maximum turnover rate ($k_{\text{cat}} = 0.72 \pm 0.05 \text{ min}^{-1}$) and the 29-mer peptide had an 87-fold faster turnover ($k_{\text{cat}} = 29 \pm 2 \text{ min}^{-1}$) than that for HupB_*Mtb* ($k_{\text{cat}} = 0.33 \pm 0.02 \text{ min}^{-1}$). The relatively high affinity of Eis for HupB and histones and a rather low apparent efficiency of acetylation of these substrates by Eis likely occur because the HupB and histones contain binding surfaces for Eis not present in the Lys-rich peptide substrates, which when interacting with Eis do not support or perhaps even prevent efficient acetylation of the Lys-rich tail regions or Lys residues in the core region. In the cell, these surfaces may be engaged in interactions with nucleic acids or other proteins and would not be exposed for Eis binding. The complexity of the HupB/histone acetylation mechanism by Eis *in vivo* is an intriguing subject of future research.

Eis_*Msm* acetylated H1_*Bta* at a 2-fold slower rate when compared to Eis_*Mtb*. Eis_*Mab* and Eis_*Tpa* acetylated H1_*Bta* 2- and 2.8-fold faster than Eis_*Mtb*, respectively. The fact that Eis homologues from other bacteria can also acetylate H1_*Bta* with comparable efficiency suggests that this control of gene expression and nucleoid architecture is conserved among Eis- and HupB-containing bacteria. While other AACs have been documented to acetylate histone-like proteins (e.g., AAC(6')-Ii⁴⁸ and the chromosomal AAC(6')-Iy⁴⁹ that shows structural similarity to histone acetyltransferase), none of the AACs tested herein were able to acetylate HupB (Figure S4). This suggests that Eis is unique in its ability to acetylate HupB.

Our crystal structure of Eis–HupB peptide complex showed that the (P)AKK region of the peptide was bound in the substrate-binding site of the peptide. We previously observed tobramycin³⁶ and our Eis inhibitors^{32,35,42} in this binding pocket (Figure 6). Despite structural differences in the substrate binding pockets, other GCN5 family acetyltransferases such as HAT GCN5 enzyme from *Tetrahymena* (tGCN5) bind peptides so that the substrate lysine is positioned in the active site relative to the GCN5 fold similarly to Eis (Figure S3).
50–52

This and our previous structures show that the potent inhibitors of Eis acetylation of KAN that we recently developed^{32,35,42} bind Eis at the same site as the peptide or KAN, indicating that the inhibitors may be competitive with peptide acetylation. As we expected, representative potent inhibitors of KAN acetylation by Eis also potently inhibited acetylation of the lysine-rich C-terminal region of HupB by Eis. These inhibitors will be used as specific probes of Eis acetylation of HupB and, potentially, of other proteins in the mycobacterial cell.

The kinetics of the NAD⁺-dependent deacetylation of AcHupB_*Mtb* by Rv1151c ($K_m = 4.7 \pm 0.6 \mu\text{M}$, $k_{\text{cat}} = 1.27 \pm 0.06 \text{ min}^{-1}$, Figure 5) are comparable to the acetylation kinetics of this protein by Eis and are in good agreement with those previously reported for the deacetylation of the acetylated acetyl-CoA synthetase ($K_m < 4 \mu\text{M}$; $k_{\text{cat}} = 1.2 \pm 0.06 \text{ min}^{-1}$).

²⁵ These data demonstrate that Rv1151c is a deacetylase with little substrate specificity and that Rv1151c likely deacetylates HupB *in vivo* to regulate its acetylation status together with Eis.

The extensive acetylation of HupB_*Mtb* by Eis suggests that, like mammalian histones, acetylation sites of HupB_*Mtb* may serve as epigenetic markers that may play a role in DNA replication, gene expression, and regulation of the cell growth. While there were acetylation events outside of the tail, which based on sequence alignments with other Hu proteins (Figure S1) starts near residue 90 for HupB, this is likely due to the acetylation taking place *in vitro* and may be acetylated only marginally by Eis and *in vivo* acetylation of these sites may be nonexistent. This mechanism may have evolved in this bacterium as a means to withstand the stresses of the human immune system. Disabling this mechanism by inhibitors may be useful for increasing vulnerability of *Mtb* to the immune system and antibiotics. Studies are currently underway in our groups to test this hypothesis.

CONCLUSION

In summary, this study described the acetylation of a histone-like nucleoid mycobacterial protein HupB by acetyltransferase Eis and established that HupB deacetylation can be carried out by a deacetylase Rv1151c. These findings suggest that these two opposing activities may regulate the acetylation status of HupB *in vivo*, in turn controlling chromosome organization in the pathogen *M. tuberculosis*. We also presented the crystal structure of Eis in complex with a lysine-rich peptide from a histone tail-like region of HupB, consistent with the observed acetylation pattern. This study provided an indication that histone-like protein acetylation in prokaryotes may be similarly regulated to histone acetylation in prokaryotes that have evolved to respond to stresses, such as *M. tuberculosis* responding to immune stresses of the host cell by toggling “on” and “off” its replication status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Bta	Bos taurus
Eis	enhanced intracellular survival

HAT	histone acetyltransferase
HDAC	histone deacetylase
KAN	kanamycin
Mab	Mycobacterium abscessus
MDR	multidrug resistant
Msm	Mycobacterium smegmatis
Mtb	Mycobacterium tuberculosis
NAP	nucleoid-associated protein
TB	tuberculosis
TCA	trichloroacetic acid
Tpa	Tsukamurella paurometabola
XDR	extensively drug resistant

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Figure 1.

Sequence alignment of HupB_Mtb with the three isoforms of H1_Bta (H1.1, H1.2, and H1.3). The lysine residues acetylated by Eis are indicated by green rectangles (HupB_Mtb), yellow rectangles (H1.1_Bta), red rectangles (H1.2_Bta), or blue rectangles (H1.3_Bta). Previously published acetylated lysines of HupB_Mtb are indicated by pale green (Eis catalyzed) or pale gray (other acetyltransferase catalyzed) ovals.¹⁹ The gray rectangle indicates the only residue acetylated prior to reaction with Eis. Asterisks indicate that the modification was found in HupB_Mtb isolated from Mtb H37Rv without any treatment with Eis.

Figure 2. Michaelis–Menten curves for the acetylation by Eis of (A) histones from *Bos taurus* (H1_Bta), (B) the HupB_Mtb 29-mer peptide, and (C) the full-length HupB_Mtb. The best-fit kinetic parameters (K_m , k_{cat} , and k_{cat}/K_m) are listed in the graphs.

Figure 3.
Time courses of acetylation of H1_ *Bta* by Eis enzymes from four bacterial species containing HupB-like proteins.

Figure 4.

(A) Structures of three known inhibitors of Eis aminoglycoside acetylation. (B) A bar graph showing inhibition of the HupB_ *Mtb* 29-mer peptide acetylation by the molecules presented in panel A. (C) Inhibition of the HupB_ *Mtb* 29-mer peptide with varying concentrations of compound **2**, indicating a dose-dependence.

Figure 5.

Kinetics of HupB deacetylation and salt concentration dependence on HupB acetylation kinetics. (A) The Michaelis–Menten curve for the deacetylation of AcHupB_*Mtb* by Rv1151c. The best-fit kinetic parameter values are given in the graph. (B) The time course of acetylation of HupB_*Mtb* by Eis at two different salt concentrations.

Figure 6.

Structures of Eis in complex with a HupB peptide and other ligands. (A) The structure of a monomer of Eis in complex with CoA and a HupB_*Mtb* 9-mer peptide (PDB ID: 6B0U). (B) A zoom-in view of the Eis substrate binding site bound to the HupB peptide. Eis residues interacting with the peptide are shown as orange sticks. The $mF_o - DF_c$ polder omit map contoured at 3.5σ is shown by a brown mesh. (C and D) Structures of Eis in complex with tobramycin in a 6'-acetylation conformation (PDB ID: 4JD6, panel C) and in complex with an Eis inhibitor (**11c**) (PDB ID: 5EBV,³² panel D). Note: CoA and other ligands are shown as sticks with the following atom color scheme: C is in light blue (for ligands) or green (for CoA), N is dark blue, O is in red, S and P are in yellow.