Succinylation Analysis Reveals the Involvement of Lysine Succinylation in Metabolism in Pathogenic Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis, remains one of the most prevalent human pathogens and a major cause of mortality worldwide. Metabolic network is a central mediator and defining feature of the pathogenicity of Mtb. Increasing evidence suggests that lysine succinylation dynamically regulates enzymes in carbon metabolism in both bacteria and human cells; however, its extent and function in Mtb remain unexplored. Here, we performed a global succinylation analysis of the virulent Mtb strain H37Rv by using high accuracy nano-LC-MS/MS in combination with the enrichment of succinylated peptides from digested cell lysates and subsequent peptide identification. In total, 1545 lysine succinylation sites on 626 proteins were identified in this pathway. The identified succinylated proteins are involved in various biological processes and a large proportion of the succinylation sites are present on proteins in the central metabolism pathway. Site-specific mutations showed that succinylation is a negative regulatory modification on the enzymatic activity of acetyl-CoA synthetase. Molecular dynamics simulations demonstrated that succinylation affects the conformational stability of acetyl-CoA synthetase, which is critical for its enzymatic activity. Further functional studies showed that CobB, a sirtuin-like deacetylase in Mtb, functions as a desuccinylase of acetyl-CoA synthetase in in vitro assays. Together, our findings reveal widespread roles for lysine succinylation in regulating metabolism and diverse processes in Mtb. Our data provide a rich resource for functional analyses of lysine succinylation and facilitate the dissection of metabolic networks in this life-threatening pathogen. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045922, 796–811, 2015.

Post-translational modifications (PTMs)1 are complex and fundamental mechanisms modulating diverse protein properties and functions, and have been associated with almost all known cellular pathways and disease processes (1, 2). Among the hundreds of different PTMs, acylations at lysine residues, such as acetylation (3–6), malonylation (7, 8), crotonylation (9, 10), propionylation (11–13), butyrylation (11, 13), and succinylation (7, 14–16) are crucial for functional regulations of many prokaryotic and eukaryotic proteins. Because these lysine PTMs depend on the acyl-CoA metabolic intermediates, such as acetyl-CoA (Ac-CoA), succinyl-CoA, and malonyl-CoA, lysine acylation could provide a mechanism to respond to changes in the energy status of the cell and regulate energy metabolism and the key metabolic pathways in diverse organisms (17, 18).

Among these lysine PTMs, lysine succinylation is a highly dynamic and regulated PTM defined as transfer of a succinyl group (-CO-CH2-CH2-CO-) to a lysine residue of a protein molecule (8). It was recently identified and comprehensively validated in both bacterial and mammalian cells (8, 14, 16). It was also identified in core histones, suggesting that lysine succinylation may regulate the functions of histones and affect chromatin structure and gene expression (7). Accumulating evidence suggests that lysine succinylation is a widespread and important PTM in both eukaryotes and prokaryotes and regulates diverse cellular processes (16). The system-wide studies involving lysine-succinylated peptide

1 The abbreviations used are: TB, Tuberculosis; Mtb, Mycobacterium tuberculosis; PTM, post-translational modification; Acs, acetyl-CoA synthetase; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SPC, simple point charge; LINCS, linear constraint solver; PME, particle mesh Ewald; MD, molecular dynamics; Ac-CoA, acetyl-CoA; CoA, Coenzyme A; NAD+, nicotinamide adenine dinucleotide; NAM, Nicotinamide; Sir15, siruin; RMSD, root mean square deviation; Rgs, gyrations; RMSF, residue-specific root mean square fluctuation.

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immunoprecipitation and liquid chromatography-mass spectrometry (LC-MS/MS) have been employed to analyze the bacteria (E. coli) (14, 16), yeast (S. cerevisiae), human (HeLa) cells, and mouse embryonic fibroblasts and liver cells (16, 19). These succinylome studies have generated large data sets of lysine-succinylated proteins in both eukaryotes and prokaryotes and demonstrated the diverse cellular functions of this PTM. Notably, lysine succinylation is widespread among diverse mitochondrial metabolic enzymes that are involved in fatty acid metabolism, amino acid degradation, and the tricarboxylic acid cycle (19, 20). Thus, lysine succinylation is reported as a functional PTM with the potential to impact mitochondrial metabolism and coordinate different metabolic pathways in human cells and bacteria (14, 19–22).

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), is a major cause of mortality worldwide and claims more human lives annually than any other bacterial pathogen (23). About one third of the world’s population is infected with *Mtb*, which leads to nearly 1.3 million deaths and 8.6 million new cases of TB in 2012 worldwide (24). *Mtb* remains a major threat to global health, especially in the developing countries. Emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) *Mtb*, and also the emergence of co-infection between TB and HIV have further worsened the situation (25–27). Among bacterial pathogens, *Mtb* has a distinctive life cycle spanning different environments and developmental stages (28). Especially, *Mtb* can exist in dormant or active states in the host, leading to asymptomatic latent TB infection or active TB disease (29). To achieve these different physiologic states, *Mtb* developed a mechanism to sense diverse signals from the host and to coordinately regulate multiple cellular processes and pathways (30, 31). *Mtb* has evolved its metabolic network to both maintain and propagate its survival as a species within humans (32–35). It is well accepted that metabolic network is a central mediator and defining feature of the pathogenicity of *Mtb* (23, 36–38). Knowledge of the regulation of metabolic pathways used by *Mtb* during infection is therefore important for understanding its pathogenicity, and can also guide the development of novel drug therapies (39). On the other hand, increasing evidence suggests that lysine succinylation dynamically regulates enzymes in carbon metabolism in both bacteria and human cells (14, 19–22). It is tempting to speculate that lysine succinylation may play an important regulatory role in metabolic processes in *Mtb*. However, to the best of our knowledge, no succinylated protein in *Mtb* has been identified, presenting a major obstacle to understand the regulatory roles of lysine succinylation in this life-threatening pathogen.

In order to fill this gap in our knowledge, we have initiated a systematic study of the identities and functional roles of the succinylated protein in *Mtb*. Because *Mtb* H37Rv is the first sequenced *Mtb* strain (40) and has been extensively used for studies in dissecting the roles of individual genes in pathogenesis (41), it was selected as a test case. We analyzed the succinylome of *Mtb* H37Rv by using high accuracy nano-LC-MS/MS in combination with the enrichment of succinylated peptides from digested cell lysates and subsequent peptide identification. In total, 1545 lysine succinylation sites on 626 proteins were identified in this pathogen. The identified succinylated proteins are involved in various biological processes and render particular enrichment to metabolic process. A large proportion of the succinylation sites are present on proteins in the central metabolism pathway. We further dissected the regulatory role of succinylation on acetyl-CoA synthetase (Acs) via site-specific mutagenesis analysis and molecular dynamics (MD) simulations showed that reversible lysine succinylation could inhibit the activity of Acs. Further functional studies showed that CobB, a sirtuin-like deacetylase in *Mtb*, functions as a deacytelylase and as a desuccinylase of Acs in *in vitro* assays. Together, our findings provide significant insights into the range of functions regulated by lysine succinylation in *Mtb*.

**EXPERIMENTAL PROCEDURES**

* Bacterial Strains and Growth Conditions—*The *M. tuberculosis* strain H37Rv (ATCC 27294) was obtained from the National Center for Medical Culture Collections (CMCC, Beijing, China). The seed culture was grown to log phase (OD600 ~ 1.2) in Middlebrook 7H9 liquid medium (Difco) supplemented with ADC (Albumin-dextrose-catalase) at 37 °C. After 7 days of growth of log phase, the cultures were pellet, washed twice with PBS (0.1 M Na2HPO4, 0.15 M NaCl, pH 7.5), and resuspended in fresh liquid medium following by incubation for 2–3 weeks to a final OD600 ~ 0.6–0.8. For carbon source utilization studies, *Mtb* seed culture was resuspended in fresh complex growth media containing additional carbon sources, such as 0.2% pyruvate, 0.2% glucose, 0.2% glycerol, 0.2% succinate, or 0.2% acetate, respectively.

* Preparation of Protein Lysate and In-solution Trypsin Digestion—*To identify as many succinylation events as possible, we have performed a series of preliminary experiments using different amount of *Mtb* proteins to find the best protocol. Following is the optimized experimental procedures based on these preliminary experiments. In brief, bacterial cells (250 ml) were harvested by centrifugation at 2500 × g for 10 min at 4 °C. The pellet was washed twice with ice-cold PBS, resuspended in ice-cold PBS containing 1× protease inhibitor mixture (Roche Diagnostics Ltd, Mannheim, Germany) and 1× PMSE (Beyotime Institute of Biotechnology, Jiangsu, China). The mixture was placed in Lysing Matrix B bead beater vials (pre-filled with 0.1 mm silica; MP Biomedicals, Santa Ana, CA) and homogenized using the FastPrep-24 sample preparation system (MP Biomedical, CA). The cells were lysed by bead-beating for five cycles of 35 s at a setting of 6.5 m/s. Between each cycle of bead beating, the samples were incubated on ice for 3 min. Cell lysates were cleared of silica and cellular debris via centrifugation at 12,000 × g for 15 min at 4 °C. Supernatant was then filtered through the 0.2 µm syringe filters twice to sterilize lysis. The clarified lysate was precipitated by using 10% trichloroacetic acid and 1% sodium deoxycholate, and then washed twice with ice cold acetone. The precipitated proteins (2 mg) were redissolved in 50 µl ammonium bicarbonate, then in-solution digested by trypsin according to previously described (14, 42). The protein concentration was determined with the Bradford assay (Bio-Rad, Hercules, CA).
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**Immunoadfinity Enrichment of Lysine Succinylated Peptides**—Succinylated peptides were enriched using agarose-conjugated anti-succiny l lysine antibody (PTM Biolabs Inc., Chicago, IL) as previously described (14). Briefly, tryptic peptides were redissolved in NETN buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and then incubated with anti-succinyllysine antibody conjugated protein A agarose beads at 4 °C for 6 h with gentle rotation. The supernatant was removed and the beads were washed three times with NETN buffer, twice with ETN buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA), followed by three times wash with water. The bound peptides were eluted by washing three times with 1% trifluoroacetic acid. The eluates were combined and dried in a SpeedVac. The resulting succinylated peptides were loaded onto self-packed C18 STAGE tips according to the manufacturer’s instructions to desalt the sample, prior to nano-HPLC-MS/MS analysis.

**Nano-HPLC-MS/MS Analysis**—The enriched peptides were dissolved in the HPLC buffer A (0.1% (v/v) formic acid in water), and analyzed by online nanoflow LC-MS/MS using an easy nLC-1000 system (Thermo Scientific) connected to a Q-Exactive (Thermo Scientific) mass spectrometer. Briefly, samples were injected onto the analytical C18-nanocapillary LC column (5 μm particle size, 100 Å pore diameter) and eluted at a flow rate of 300 nL/min with a 45 min gradient from 5% solvent B (90% ACN/0.1% formic acid, v/v) to 80% solvent B (90% ACN/0.1% formic acid, v/v) to 80% solvent B. The peptides were then directly ionized and sprayed into a Q-Exactive mass spectrometer by a nanospray ion source. Mass spectrometer was operated in a data-dependent mode with an automatic switch between MS and MS/MS acquisition. Full MS spectra from m/z 350 to 1600 were acquired with a resolution of 70,000 at m/z = 200 in profile mode. Following every survey scan, up to 15 most intense precursor ions were picked for MS/MS fragmentation by higher energy C-trap dissociation (HCD) with normalized collision energy of 35%. Lock mass at m/z 445.12003 was enabled for full MS scan. The dynamic exclusion duration was set to 5 s with a repeat count of one and ± 10 ppm exclusion window.

**Protein Sequence Database Search**—All acquired raw data were processed with the MaxQuant software (version 1.3.0.5) (43). The peak lists were searched against the Mtb H37Rv protein database from NCBI ftp site (http://www.ncbi.nlm.nih.gov/) containing 4018 protein sequences. Two missed cleavages were allowed for trypsin. The precursor and fragment ion mass tolerances were 10 ppm and 0.02 Da, respectively. Carbamidomethylation (Cys) was set as a fixed modification, whereas oxidation (Met), deamidation (Asn/Gln), succinylation (lysine) and acetylation (lysine) (Protein N-terminal) were set as variable modifications. Minimum peptide length was set at six. The estimated false discovery rate (FDR) thresholds for modification site, mass gradient from 5% solvent B (90% ACN/0.1% formic acid, v/v) to 80% solvent B (90% ACN/0.1% formic acid, v/v) to 80% solvent B. The peptides were then directly ionized and sprayed into a Q-Exactive mass spectrometer by a nanospray ion source. Mass spectrometer was operated in a data-dependent mode with an automatic switch between MS and MS/MS acquisition. Full MS spectra from m/z 350 to 1600 were acquired with a resolution of 70,000 at m/z = 200 in profile mode. Following every survey scan, up to 15 most intense precursor ions were picked for MS/MS fragmentation by higher energy C-trap dissociation (HCD) with normalized collision energy of 35%. Lock mass at m/z 445.12003 was enabled for full MS scan. The dynamic exclusion duration was set to 5 s with a repeat count of one and ± 10 ppm exclusion window. After washing three times with PBS and incubated with the whole cell lysates overnight at 4 °C. The beads were then washed three times with PBS to remove the unbound proteins. Bound proteins were boiled in SDS loading buffer for 5 min, then subjected to 15% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. For Western blotting, the membrane was blocked overnight at 4 °C or 37 °C for 2 h in TBS buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 5% bovine serum albumin (BSA) and incubated with either the specific Mtb protein antibody or the anti-succinyl lysine antibody (PTM Biolabs Inc., Chicago, IL) (1:2000, in TBS/5% nonoate) overnight at 4 °C. After washing three times with TBS buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000 dilutions) for 1 h at 37 °C. The membrane was then washed with TBS buffer and visualized with enhanced chemiluminescence (ECL) immunoblotting detection reagents (Advansta Inc., Menlo Park, CA). The density of each band was determined with a fluorescence scanner (ImageQuant TL, GE Healthcare). Densitometry analysis was performed via the ImageJ suite (http://rsbweb.nih.gov/ij/).

Site-directed Mutagenesis and Purification of AcS—The deacetylase (CobB) and AcS expression vectors (pET32a-cobB and pET20b-acS, kindly provided by Professor Jiao-Yu Deng, Wuhan Institute of Virology, CAS) were transformed into E. coli BL21 (DE3) for protein expression. The site-directed mutation of acs gene was introduced into the selected sites by PCR reaction. The PCR product was transformed into E. coli BL21 (DE3), and subsequently the mutagenesis was completely sequenced to confirm the presence of the site-directed mutation. Mutagenic primers were given below with the mutated base triplets underlined: K193R-sense: 5′-CGCGGAGAC-
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CATGCCCCTCA AGCCGGCC-3', K193R-antisense: 5'-CGATGG-
TCTGCGCGGCGAATGCCTGCT-3', K366R-sense: 5'-TTAT-
TGGTGGATGGCCTGATGCCCGGAGGAC-3', K366R-antisense: 5'-
GCACCTCATAAACATCCGGATGAGGTT-3'. The wild type E. coli BL21 (DE3) overexpresses a recombinant Acs protein, which was used in this study.

The reaction products were analyzed by SDS-PAGE and Western blotting. The purified recombinant Cbb and Acs were incubated in 50 μl of reaction buffer (50 mM Tris-HCl, pH 8.0, 600 mM NaCl, and 30 mM MgCl₂, 2 mM DTT), followed by addition of equal volume of stop solution [2.5% (w/v) FeCl₃ in 2 M HCl, 10% (w/v) trichloroacetic acid]. The reaction products were centrifuged to remove turbidity, and then measured at 540 nm.

In Vitro Desuccinylation Activity Assay—The purified recombinant CbbB and Acs were incubated in 50 μl of reaction buffer (50 mM Tris-HCl, pH 8.5, 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl₂) with or without 500 μM NAD⁺ and in the presence or absence of 2 mM Nicotinamide (NAM). The mixture was incubated at 25 °C for 10 h. The reaction products were analyzed by SDS-PAGE and Western blotting.

Molecular Dynamics Simulations—The initial model of the Acs module was prepared from the available crystal structure (PDB code 2P20, 50.56% sequence identity) via structural modeling using CPHmodels 3.2 Server (http://www.cbs.dtu.dk/services/CPHmodels). All simulations were based on the initial model and a total of seven systems were prepared for simulation using GROMACS 4.5.5 (58) in conjunction with the OPLS-AA/L all-atom force field (59). The protein was then solvated in simple point charge (SPC) water molecules in a cubic box (60), with the box edges ~1.0 nm from any atom of the protein, and eighteen additional Na⁺ ions were added to neutralize the charge of each system. Each system was then energy minimized using a steepest descents integrator either until the maximum force was less than 1000 kJ/mol/nm or until additional steps resulted in a potential energy change of less than 1 kJ/mol. Next, the simulations were performed under a constant temperature of 300 K and the V-rescale algorithm (61) was used with a temperature coupling time constant of 0.1 ps. All bond lengths were constrained using the linear constraint solver (LINCS) algorithm (62). Van der Waals interactions used a simple cut off at 1.4 nm and long-range electrostatic interactions were handled using the particle mesh Ewald (PME) method (63, 64) with a fourth-order spline interpolation and a 0.1 nm Fourier grid spacing. Once each system was sufficiently equilibrated around the target temperature, isotropic was used for pressure coupling and the constant pressure was set to 1.0 bar in all directions and a pressure coupling time constant of 1.0 ps. Finally, each system was subjected to 10 ns of molecular dynamics (MD) simulation and the time step used in the simulations was 2 fs. All analyses were performed using the GROMACS suite of tools and a secondary structure recognition algorithm (DSSP) (65), which was implemented in GROMACS. The PyMOL Molecular Graphics System (version 1.7.2, http://www.pymol.org) was employed to present the structural results of this study.

RESULTS

Detection of Protein Lysine Succinylation in Mtb—To examine the diversity and relative abundance of lysine succinylation proteins from different growth conditions of Mtb, Western blotting analysis was performed using a polyclonal anti-succinyl lysine antibody (Fig. 1). The virulent Mtb strain H37Rv were grown in minimal medium, either without an additional carbon source or treated with pyruvate, glucose, glycerol, succinate, or acetate and harvested at 30 day or 60 day. Strong immunoblot signals were observed at both 30 day and 60 day (Fig. 1), indicating that lysine succinylation was abundant in H37Rv under different culture conditions. Immunoblots with succinylated or non succinylated BSA confirmed that the protein bands detected were specific (Fig. 1). However, succinylation signals were stronger when H37Rv were grown in medium containing succinate or pyruvate as a sole carbon source than those in no carbon medium (Fig. 1), suggesting that different growth media may alter the lysine succinylation profile. As expected, Mtb exhibited different growth rate in media containing different carbon source (supplemental Fig. S1). These results implied that the lysine succinylation is likely linked with metabolic changes in Mtb. Moreover, as shown in supplemental Fig. S2, we obtained a similar result as previously reported on lysine acetylation (66) when the strain was grown on different carbon source.

Enrichment and Identification of Lysine Succinylation Sites in Mtb—To identify the lysine succinylation sites in Mtb, we used a similar procedure as previously described (7, 14, 16) (Fig. 2A). In total, we identified 1561 unique modified peptides encompassing 1545 succinylation sites (class I) from 626 proteins with a FDR below 1% for modified peptides. The proteomic data showed that 61.4% of identified unique peptides (2541) across 84.5% of the identified proteins (741) were immune-enriched, indicating the highly efficient enrichment procedure were used in this study (Fig. 2B and 2C). All spectra containing succinylation were manually inspected to ensure diagnostic b- or y-ion series as previously described (44, 45). Detailed information of all identified succinylated peptides are provided in the supplemental Table S1. The raw data has
Fig. 1. Anti-lysine succinylation immunoblots showing that different carbon sources altered succinylation profiles of *Mtb* H37Rv. Coomassie blue staining of protein lysates from A 30 days and B 60 days of cultures. Coomassie blue staining was used for the loading control. Western blotting analysis of lysine succinylation level in protein lysates from C 30 days and D 60 days of cultures with a pan anti-succinyllysine antibody. Specificity of succinylation signals was validated by succinylated or non succinylated BSA assay.

Fig. 2. Profiling lysine succinylome in *Mtb* H37Rv. A, Workflow for succinylome analysis of *Mtb* H37Rv. B, Venn diagram showing the number of identified succinylated peptides. C, Venn diagram showing the number of identified succinylated proteins. D, Distribution of peptide score. E, Distribution of precursor mass deviations.
been uploaded onto a public database PeptideAtlas (http://www.peptideatlas.org) and can be accessed through the following link: http://www.peptideatlas.org/PASS/PASS00518. Annotated peptide spectra for all succinylated peptides were deposited in PeptideAtlas and supplied as supplementary Dataset. The average peptide score was 117.9402 (Fig. 2D) and the overall absolute peptide mass accuracy was 0.5752 parts per million (ppm) (standard deviation, 0.6173 ppm) (Fig. 2E), confirming the high accuracy of modified peptide data obtained from MS. Furthermore, to assess the distribution of the average modified peptide length and succinylation sites, we calculated the number of amino acids per peptide and identified modification sites per protein. Supplemental Fig. S3A showed that the average peptide length was 15 amino acids and the average site coverage per identified protein was three lysine succinylation sites (supplemental Fig. S3B). We reasoned that multiple succinylation sites on one protein may play an important role in stabilization/destabilization of protein conformation and fulfill the delicate regulatory function.

Conservation of Succinylated Proteins—To explore the evolutionary conservation of all identified lysine succinylation sites in this study, we searched the orthologs of 626 succinylated proteins of Mtb against the following previously reported succinyloymes: Escherichia coli DH10B (14), Escherichia coli BW25113 (16), Saccharomyces cerevisiae S288c (16), Homo sapiens (16), Mus musculus (16), and Drosophila melanogaster (7). The orthologs that showed the highest homology to each other in both directions were counted and reported as the percentage of the total number of proteins in all analyzed species. The percentage of Mtb homologs of succinylated proteins in diverse organisms is showed in Table I. As a result of a global homology search using the amino acid sequence of target proteins, orthologs of 205 (~32.7%) Mtb succinylated proteins can be detected in the succinyloymes of other species (supplemental Table S2). Moreover, we found that 164 and 107 Mtb succinylated proteins have succinylated homologs in bacterial and eukaryotic cell, respectively (supplemental Fig. S4). Interestingly, most succinylated proteins are involved in central carbon metabolism and lipids metabolism. These conserved succinylation events suggest that succinylation may play an important role in essential biological processes in this pathogen.

Functional Annotation and Cellular Localization of Succinylated Proteins—To further elucidate the biological functions of succinylated proteins, we performed enrichment analysis using the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Pfam database. Firstly, we investigated the GO functional classification of all the succinylated proteins based on their biological process and molecular function (Fig. 3). The classification results for biological process showed that the largest protein group of succinylated proteins was related to growth (26.60%), followed by cellular process (18.44%), metabolic process (17.04%) and response to stimulus (9.33%). Interestingly, 1.05% and 0.12% of the total succinylated proteins were involved in immune system process and signaling, respectively, which could be an important mechanism for the survival of Mtb. In the molecular function classification, most succinylated proteins were related to the binding of various targets and enzyme activity. Approximately half of the lysine succinylated proteins (50.76%) were categorized as catalytic activity-related proteins. Another large succinylated protein group is binding to targets (45.45%) (supplemental Table S3). Within the cluster of cellular localization, a total of 74.44% of the succinylated proteins was in the cytoplasmic category by the bacterial protein subcellular localization prediction program PSORTb (47). This result was also in agreement with the biological process analysis that most succinylated proteins were involved in cellular metabolism. Importantly, we also found that a sizable portion of succinylation proteins were located on cytoplasmic membrane (11.98%), cell wall (0.48%), and extracellular (0.8%). These proteins could play a role in regulating the envelope-related proteins to impact the virulence of mycobacteria.

To further elucidate the biological functions of succinylated proteins, we performed enrichment analysis using Cytoscape plugin BinGO. As shown in supplemental Figs. S5 and S6, the succinylated proteins were mostly enriched in growth (p = 9.86 × 10⁻¹⁵), and also statistically enriched in cellular metabolism process with specific enrichment in cellular ketone metabolic process (p = 3.6 × 10⁻⁷), carboxylic acid meta-
bolic process \((p = 3.86 \times 10^{-9})\), oxoacid metabolic process \((p = 3.86 \times 10^{-9})\), and organic acid metabolic process \((p = 3.86 \times 10^{-9})\) (supplemental Fig. S5 and supplemental Table S4A). Consistently, the GO enrichment analysis of molecular functions further demonstrated that many functions were enriched in our set, including catalytic activity and binding (supplemental Fig. S6 and supplemental Table S4A). We next performed cellular compartment analysis of all succinylated proteins and obtained the similar results. As expected, a significant portion of succinylated proteins were significantly enriched in membrane and cell wall (supplemental Fig. S6 and supplemental Table S4A). Moreover, proteins involved in ribosome, citrate cycle (TCA cycle), fatty acid metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, oxidative phosphorylation, glyoxylate, and dicarboxylate metabolism were also enriched, as determined through the KEGG pathway analysis (supplemental Fig. S7 and supplemental Table S4B), indicating that lysine succinylation substrates were associated with the ribosome and cellular metabolism events. The Pfam domain analysis illustrated that most succinylated substrates were enriched with the acyl-CoA dehydrogenase domain, which catalyzed the first step in the \(\beta\)-oxidation of fatty acids and catabolism of some amino acids (supplemental Fig. S7 and supplemental Table S4B). Taken together, these findings suggested that the identified succinylated proteins have a wide distribution of functions and cellular localizations and render particular enrichment to metabolic process in Mtb.

**Sequence Recognition Motifs and Local Structural Properties**—We further compared the position-specific amino acid frequencies of the surrounding sequences (six amino acids to both termini) of succinylated lysine residues with those of all lysine residues that occurred in the Mtb proteome. We found that negatively charged amino acids (glutamic acids) was drastically overrepresented at position \(-1\) using motif-X (Fig. 4A and supplemental Table S5A). Then, we compared our data sets to the reported succinylome of E. coli, showing that the succinylation pattern found in E. coli BW25113 were quite different from Mtb site-specific succinylation motifs. Interestingly, the same amino acid (glutamic acids) was preferred at position \(+1\) in E. coli DH10B. To further assess if there is significant enrichment or depletion of specific amino acids with respect to the general amino acid composition of the entire proteome, a position-specific intensity map was generated as previously described (42). The result showed a preference for polar acidic and polar basic amino acids (Fig. 4B). It can be seen that aspartic acid was most commonly found at \(+1\) and glutamic acid was most commonly found at \(-1\), \(+2\). As well, we observed a significant preference for lysine at the \(-6\) to \(-4\), and \(+5\) positions. These results indicated that succinylation preferentially occurred in lysine-rich regions and polar acidic/basic amino acid regions of proteins. We next compared these data with the patterns generated from other succinylomes. Although we did not detect a strong bias for a specific succinylation site motif in E. coli DH10B, there were modest biases for flanking amino acid residues. The different preference of amino acid residues surrounding lysine sites suggested the unique substrate preferences in Mtb. Next, we predicted the structural features of lysine succinylation sites in identified proteins with NetSurfP algorithm (55). As expected, succinylation sites were enriched on the protein surface (84.4%) as compared with 75.5% of the all lysine residue \((p = 3.079 \times 10^{-5})\). In accordance with previous report (16), the succinylated lysine was more fre-
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**A**

*E. coli BW25113*  

*E. coli DH10B*  

*M. tuberculosis H37Rv*

**B**

| Nonpolar Hydrophobic | Polar Uncharged | Polar Acidic | Polar Basic |
|----------------------|----------------|-------------|-------------|
| A                    | F              | L           | M           |
| I                    | L              | M           | P           |
| F                    | I              | L           | P           |
| L                    | M              | P           | Q           |
| M                    | P              | Q           | R           |
| Nonpolar Hydrophobic | Polar Uncharged | Polar Acidic | Polar Basic |
| A                    | F              | L           | M           |
| I                    | L              | M           | P           |
| F                    | I              | L           | P           |
| L                    | M              | P           | Q           |
| M                    | P              | Q           | R           |

**C**

Bar graph showing the fraction of lysines (%)

- **Succinylated-lysines (DH10B)**
- **All-lysines (DH10B)**
- **Succinylated-lysines (BW25113)**
- **All-lysines (BW25113)**
- **Succinylated-lysines (H37Rv)**
- **All-lysines (H37Rv)**

**Alpha-Helix**  
**Beta-strand**  
**Coil**  
**Accessibility**

- N.S.  
- p = 0.00079  
- p = 0.00985  
- p = 0.0428
Succinylation of Enzymes Involved in Central Metabolism—Increasing evidences show that lysine succinylation may play a regulatory role in metabolic pathways, similar with lysine acetylation (4, 67). We investigated the succinylation of metabolic enzymes in Mtb by mapping succinylated proteins to KEGG metabolic pathways (supplemental Table S8). As shown in Fig. 6, a large proportion of metabolic enzymes were succinylated. The fact that nearly all enzymes in major metabolic pathways are subjected to succinylation implies that this PTM may regulate cellular metabolic process at multiple levels in Mtb. For example, a large proportion of metabolic enzymes were mapped to two essential pathways for the growth and survival of Mtb, i.e., mycolic acid biosynthesis (supplemental Fig. S9A) and purine nucleotides de novo biosynthesis pathways (supplemental Fig. S9B), were identified to be succinylated.

**Fig. 4. Bioinformatical analysis of succinylation sites.** A, Sequence Logo representation of significant motifs identified by Motif-X software. The motifs with significance of $p < 0.000001$ are shown. B, Position-specific under- or over-representation of amino acids flanking the succinylation sites. Colors were plotted by using intensity map and represent the $\log_{10}$ of the ratio of frequencies within succinyl-13-mers versus nonsuccinyl-13-mers (blue shows enrichment, yellow shows depletion). C, Distribution of succinylated and nonsuccinylated lysines in protein secondary structures. Probabilities for different secondary structures (\(\alpha\) helix, beta-strand and coil) of succinylated lysine were compared with the secondary structure probabilities of nonsuccinylated lysine on all proteins identified in this study. Significance was calculated by Wilcoxon test.

**Fig. 5. The complete interaction network of identified succinylated proteins and their interacting proteins.** The succinylated proteins were grouped using functional annotation and interaction network was visualized with Cytoscape. The red node border represents the identified succinylated proteins.
Confirmation of Succinylated Proteins by Immunoprecipitation and Western blotting—Based on how well their biological functions and importance are understood, ten newly identified succinylated proteins, including AtpA, AtpF, KasA, DesA1, FadD31, Fgd1, GlcB, HspX, MtrA, and Pks2, were selected for verification. These proteins were subjected to IP and Western analysis (supplemental Fig. S10). The immunoprecipitated proteins were first confirmed by their specific antibodies. Anti-succinyl lysine antibody detected the respective proteins, confirming that they contain succinylated lysine residues. In comparison, the unmodified BSA (negative control protein) was not detected by anti-succinyl lysine antibodies, whereas the immuno-reactive signal was strongly observed when the succinylated BSA was used as positive control (supplemental Fig. S10). These data provided additional biochemical evidence for these identified succinylated proteins.

Effect of Succinylation on Acs Activity—Acs plays a crucial role in central metabolism and is important for maintaining adequate levels of Ac-CoA, a metabolite of many anabolic and catabolic processes. It is reported that acetylation of Acs completely abolishes the conversion of acetate to Ac-CoA (57, 68, 69) (Fig. 7A). In this study, we identified two reliable succinylation sites on Acs, Lys193 and Lys366 (Fig. 7B). We speculate that the Acs activity may be regulated by reversible succinylation. To test our hypothesis, the acs gene of Mtb was cloned and was overexpressed as a His-tagged fusion protein, which was purified by Ni2+/H100 chromatography. As shown in Fig. 7C, the recombinant Acs were both acetylated and succinylated. Based on the conservation analysis, lysine at 193 (in coil) and 366 (in α helix) positions of Acs were conserved in the Mtb orthologs, suggesting that these residues could be important for an evolutionarily conserved function (supplemental Fig. S11A). To determine the functional consequences of succinylation on Acs activity, we converted the modified residues at the two positions (Lys193 and Lys366) to arginine (R), which essentially locks Acs into a constitutively desuccinylated state. All mutations were verified by both DNA sequencing (supplemental Fig. S11B) and MS analysis.
Then, we measured the enzymatic activity of Acs and its mutants. The results showed that mutation of Lys193 and Lys366 to R (K193R, K366R, K193R/K366R), which conserved the positive charge and structure with unmodified lysine (15), led to a significant increase of the enzymatic activity (p < 0.01) (Fig. 7D). These data suggest that succinylation on Lys193 and Lys366 was a negative regulatory modification on Acs activity.

**NAD⁺ Dependent Desuccinylase Activity of Mtb**—The sirtuin proteins, which are one conserved family of deacetylases, have a unique ability to remove acetyl and other acyl groups from protein lysine side chains in a nicotinamide adenine...
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Succinylome Analysis of Mycobacterium tuberculosis

Among all metabolic pathways, mycolic acid biosynthesis pathway is unique to mycobacteria and is crucial for their success as pathogens (74). Our data showed that a large proportion of enzymes in mycolic acids biosynthesis pathway were succinylated (supplemental Fig. S9A). For example, the single fatty acid synthase (FAS) I type protein FAS, which generates CoA esters from Ac-CoA primers, creating precursors for elongation by all other fatty acid and polyketide systems, was found to be succinylated. In FAS-II system of mycolic acid biosynthesis, sets of enzymes, which can be inhibited by many drugs used in treatment of TB (75), were also succinylated. Therefore, the reversible succinylation, although the mechanism remains unknown, could be an important mechanism for regulating the interaction of these drugs with FAS-II-related enzymes, and could also be targets for novel drug therapies. The recent observation showed that Mtb is metabolically flexible and can even alter the host cellular metabolism, such as the pathogen-induced dysregulation of host lipid synthesis, to create a niche that is ideally suited to its persistent lifestyle (76). Mtb can utilize the lipid bodies accumulated in the infected host as a nutrient source via $\beta$-oxidation pathway and this seems to sustain intracellular Mtb in a physiological state. Thus, aside from fatty acid biosynthesis pathway, the $\beta$-oxidation of fatty acids, which serves as carbon and energy source, is also required for survival of Mtb in the infected host cell (77). Several enzymes involved in this pathway were found to be succinylated in this study (Fig. 6), indicating that lysine succinylation may play a crucial role in regulating the fatty acid metabolism of Mtb during infection.

dinucleotide (NAD$^+$)-dependent manner (70). Recently, the human sirtuin protein (Sir5) was showed to be an efficient NAD$^+$-dependent protein lysine desuccinylase and de-malonylase (8, 71). In E. coli, a sirtuin-like protein CobB has deacetylase/desuccinylase activity (14). In Mtb H37Rv, the sirtuin-like protein, CobB (Rv1151c) is able to deacetylate Acs (57, 69). Thus, CobB may have desuccinylase activity in Mtb H37Rv. To test this possibility, we expressed and purified CobB to measure the protein desuccinylase activity in the presence of NAD$^+$. As shown in Fig. 7E and 7F, CobB can desuccinylate Acs, while no reduction of succinylation was observed when CobB or NAD$^+$ was withdrawn from the reaction. Consistent with previous reports, CobB can also deacetylate Acs with similar efficiency (69, 72). Therefore, our data suggest that CobB is a bifunctional enzyme with both lysine desuccinylase and deacetylation activities in Mtb.

**Molecular Dynamics Simulations of Acs**—To gain insight into potential mechanisms by which succinylation might affect Acs enzymatic activity, we used molecular dynamics (MD) simulations to determine how succinylation of K193 and K366 influences the structure of Acs. It is reported that Acs uses the CoA as substrate to form the Ac-CoA complex and the residues in Acs that interact with the CoA may affect the binding of Acs with CoA by changing the conformation (73). In this study, we found the CoA-binding region marked with circular of Acs with CoA by changing the conformation (73).

Among all metabolic pathways, mycolic acid biosynthesis pathway is unique to mycobacteria and is crucial for their success as pathogens (74). Our data showed that a large proportion of enzymes in mycolic acids biosynthesis pathway were succinylated (supplemental Fig. S9A). For example, the single fatty acid synthase (FAS) I type protein FAS, which generates CoA esters from Ac-CoA primers, creating precursors for elongation by all other fatty acid and polyketide systems, was found to be succinylated. In FAS-II system of mycolic acid biosynthesis, sets of enzymes, which can be inhibited by many drugs used in treatment of TB (75), were also succinylated. Therefore, the reversible succinylation, although the mechanism remains unknown, could be an important mechanism for regulating the interaction of these drugs with FAS-II-related enzymes, and could also be targets for novel drug therapies. The recent observation showed that Mtb is metabolically flexible and can even alter the host cellular metabolism, such as the pathogen-induced dysregulation of host lipid synthesis, to create a niche that is ideally suited to its persistent lifestyle (76). Mtb can utilize the lipid bodies accumulated in the infected host as a nutrient source via $\beta$-oxidation pathway and this seems to sustain intracellular Mtb in a physiological state. Thus, aside from fatty acid biosynthesis pathway, the $\beta$-oxidation of fatty acids, which serves as carbon and energy source, is also required for survival of Mtb in the infected host cell (77). Several enzymes involved in this pathway were found to be succinylated in this study (Fig. 6), indicating that lysine succinylation may play a crucial role in regulating the fatty acid metabolism of Mtb during infection.
It is now widely accepted that metabolic adaptation to the host environment is a defining feature of the pathogenicity of Mtb (38, 78). However, we lack biochemical knowledge of its metabolic networks and how they are regulated. The succinyl-CoA is a succinyl donor to lysine and the concentration of succinyl-CoA may regulate succinylation levels globally. Our understanding of the role of succinyl-CoA and lysine succinylation on the control of Mtb metabolism remains rudimentary. However, their importance in the control of metabolism in bacteria, yeast and human, and the conservation of the enzymes involved in these pathways in Mtb, suggest that lysine succinylation may work together with many other PTMs to regulate the metabolic pathways of Mtb. Mtb may have unique mechanisms involved in controlling metabolic flux via succinyl-CoA and protein succinylation, because it resides in humans as its only known natural host and a large part of the energy status in Mtb occurs through the interaction with the human cells (35). Reversible succinylation is a strong candidate for dynamically controlling metabolic pathways and enabling Mtb to adapt to the changing host environment. To better understand the role of
succinyl-CoA and protein succinylation in Mtb metabolism, it is essential to acquire an in-depth description of the succinylome in Mtb. Furthermore, it is important to understand how the succinylation of metabolic enzymes regulates metabolic pathways in Mtb. More studies need to be done to accomplish this. Firstly, examine how environmental conditions as well as the carbon and energy balance of Mtb cells can influence the level of lysine succinylation. Secondly, determine the effects of succinylation on enzymatic activity. Thirdly, identify lysine succinyltransferase and desuccinylase involved in controlling the succinylation status of proteins in Mtb. In mammalian cell, the histone H3 propionylation might be generated by the histone acetyltransferase p300 and that selection of donor molecules (propionyl-CoA versus acetyl-CoA) may determine the difference of modifications (79). In this study, we identified the first desuccinylase, CobB, in Mtb, however, it remains unknown whether succinyltransferase and more desuccinylases exist in Mtb. Acetylation of Acs by the acetyltransferase (Pat) inactivated the enzyme in S. enterica, whereas deacetylation by the CobB reactivated it (80). We found the Pat homolog in Mtb (Rv0998) and further studies are thus necessary to identify the succinyltransferase in Mtb.

Being only one of many PTMs, lysine succinylation competes with other modifications, such as acetylation, for the same lysine residues. Accumulating evidence indicated that lysine acetylation is a conserved PTM that links Ac-CoA metabolism and cellular signaling (81, 82). Acs is the enzyme catalyzes the conversion of acetate into Ac-CoA and the level of Ac-CoA can fluctuate according to the changing metabolic conditions (83). In this study, we demonstrated that the enzymatic activity of Acs is affected by the lysine succinylation. It is thus expected that crosstalk exists between protein succinylation and acetylation mediated by the Ac-CoA can fluctuate according to the changing metabolic pathways (84). In both Mtb and human cells. Jungblut and Schlüter proposed a systematic nomenclature for the comprehensive description of protein species and suggested that protein species sequence including PTMs should replace the basic unit protein sequence (84, 85). Therefore, our succinylome data may help to provide the accurate and detailed description of protein species in Mtb and to elucidate potential biological roles of each protein species.

In summary, we report the first large-scale, high resolution MS-based survey of lysine succinylation in Mtb. The dataset presented may serve as important starting point for assessing the functional relevance of succinylation sites in this life-threatening pathogen. Given that the high abundance of lysine succinylation and its induced conformation fluctuation, it is likely that reversible succinylation may be an important regulatory mechanism in metabolism. It would be interesting and important to investigate the biological functions of reversible succinylation in metabolic regulation in Mtb.

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