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Original Article

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Lysine acetylation regulates the activity of *Escherichia coli* S-adenosylmethionine synthase

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Abstract

Lysine acetylation is one of the most abundant post-translational modifications. However, physiological roles of this modification in bacteria are largely unknown. Previous protein acetylome analysis showed that *Escherichia coli* adenosylmethionine synthase (MAT) undergoes acetylation *in vivo*, but the biological functions of this modification still need to be uncovered. In this study, MAT of *E. coli* was over-expressed and purified. Subsequent mass spectrometry analysis showed that 12 lysine residues of the protein were acetylated. Site-directed mutagenesis analysis was performed and the results showed that acetylated lysine residues play important roles in the enzymatic activity of MAT. Next, deacetylation assay was performed by using CobB as the deacetylase, and the results showed that CobB could deacetylate MAT *in vitro*. In addition, the enzymatic activities of acetylated and deacetylated MAT were compared *in vitro*, and results showed that acetylation led to a decrease in its enzymatic activity, which could be reversed by CobB deacetylation. Altogether, our data suggest that CobB modulates the enzymatic activity of *E. coli* MAT *in vitro*.

Key words: S-adenosylmethionine synthase, lysine acetylation, CobB, deacetylation

Introduction

Lysine acetylation of proteins is one of the most prevalent post-translational modifications (PTMs) in both eukaryotes and prokaryotes and regulates thousands of proteins in diverse biological processes [1–12]. Nowadays, the mechanisms of bacterial proteins lysine acetylation can occur by two distinct ways [13]. The conventional mechanism relies on lysine acetyltransferase (KAT) and transfers the acetyl group from the donor such as acetyl-coenzyme A (Ac-CoA) to the ε-amino group of lysine residue enzymatically [5,14–17]. The best known acetyltransferase in *Escherichia coli* is YfiQ, also named as Pat [18,19]. In contrast, the second mechanism is non-enzymatic, which is the predominant mechanism in *E. coli* [13]. Acetyl phosphate (AcP) directly donates its acetyl group to the lysine ε-amino group [12,20]. Lysine acetylation of proteins in *E. coli* depends largely on a non-enzymatic AcP-dependent mechanism [21]. Acetylation can be enzymatically reversed by lysine deacetylases (KDACs). In *E. coli*, the best characterized deacetylase is CobB, which shows no preference for enzymatic or non-enzymatic lysine acetylation substrate sites [22].

S-adenosyl-L-methionine (AdoMet or SAM) was identified by Cantoni in 1953 [23]. Because of its ability of donating all the groups surrounding the sulfur atom, SAM is highly reactive and participates in numerous metabolic pathways [24]. Previous studies have demonstrated that SAM can affect cellular proliferation, apoptosis, polymine

formation, and other cellular functions [25–27]. Moreover, SAM is widely used as an intracellular methyl donor in a variety of methylation reactions, which transfers methyl group to various acceptors like nucleic acids, proteins, and lipids, as well as to precursor molecules such as catecholamines, guanidinoacetate, and other biogenic amines [28–30]. *In vivo*, SAM can be catalyzed only through the S-adenosylmethionine synthase (MAT, also called methionine adenosyltransferase [EC 2.5.1.6]) encoded by *metK*, which is well-conserved from bacteria to eukaryotes [31]. It transfers the adenosyl portion of ATP to L-methionine, and yields SAM, pyrophosphate (PPi), and inorganic phosphate (Pi). In mammals, research has been focused on the role of MAT in the therapeutic application of cognitive and neurodegenerative diseases, liver diseases, and cancers [26,32]. In bacteria such as *E. coli* and *Salmonella typhimurium*, *metK* mutants show growth deficiencies and cell division defects [33–35].

Studies on the effects of PTMs on MAT activity began in the early 1990s. In 1992, Pajares *et al.* speculated that MAT from rats could be phosphorylated [36], which was proved in 1994 [37]. Recently, lysine acetylation was identified in human MAT IIα protein, which promotes MAT IIα ubiquitylation and its subsequent proteasomal degradation, and finally inhibits tumor cell proliferation [38]. In addition, recent data from protein acetylome analysis showed that *E. coli* MAT also undergoes acetylation *in vivo* [20,39,40] though the biological functions of this modification still need to be uncovered.

In this study, *E. coli* MAT was over-expressed and purified, then acetylated lysine residues of the protein were identified via mass spectrometry. After that, site-direct mutagenesis analysis was performed to probe the roles of acetylated lysine residues in the enzymatic activity of MAT. Furthermore, *in vitro* acetylation of MAT by Ac-CoA and deacetylation of acetylated MAT (Ac-MAT) via CobB were also performed and the enzymatic activities of acetylated and deacetylated MAT were compared.

Materials and Methods

Bacterial strains and culture condition

All of the bacterial strains in this study were listed in **Table 1**. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) broth medium (1.0% NaCl, 0.5% yeast extract, 1.0% tryptone); LB plates also contain 2.0% agar. Cell growth was monitored by detecting the absorbance at 600 nm (A_{600}). Ampicillin (100 μg/ml) or chloramphenicol (50 μg/ml) was added into the growth medium when needed. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at the concentration indicated in order to induce the expression of genes carried by various plasmids.

Table 1. Strains used in this study

Strain	Relevant characteristic(s)	Source
E. coli		
BL21 (DE3)	Host for protein expression	Laboratory collection
DH5α	Host for plasmid propagation	Laboratory collection
W3110	Wild type for measurement of intracellular SAM pool; F-λ-*IN(rrnD-rrnE)1 rph-1*	Laboratory collection
W3110 Δ*cobB*	*cobB* deletion strain for measurement of intracellular SAM pool	Laboratory collection

Cloning of *E. coli metK* gene, and site-directed mutagenesis

E. coli W3110 genomic DNA was isolated and purified using the Bacteria DNA Kit (TIANGEN, Beijing, China). *E. coli metK* was amplified from W3110 genomic DNA using Phusion™ DNA polymerase (Thermo Fisher, Waltham, USA), digested with *Sac*I and *Xba*I (Thermo Fisher), and ligated into plasmid pCA24N (a generous gift from Prof. Shengce Tao of Shanghai Jiao Tong University). Site-directed mutations were constructed by overlapping PCR. The 1.2-kb fragments containing *metK* mutant genes were cut with *Sac*I and *Xba*I and cloned into plasmid pCA24N, which was digested with the same restriction enzymes. The plasmids used were listed in **Supplementary Table S1**. All the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells for protein expressions. PCR products were purified using the Gel Extraction Kit (Omega, Norcross, USA). Plasmids were isolated using the Plasmid Mini Kit I (Omega). The sequences of oligonucleotide primers used were listed in **Supplementary Table S2**.

Recombinant protein expression and purification

Recombinant strains *E. coli* BL21 (DE3)/pCA24N-*metK*, site-directed mutants, and *E. coli* BL21 (DE3)/pMAL-c2X-*cobB* were grown to exponential phase at 37°C in LB medium plus chloramphenicol (50 μg/ml) and ampicillin (100 μg/ml), respectively. Expressions of MAT and its mutants were induced with 0.5 mM IPTG at 16°C for 14 h. Bacterial cells were harvested by centrifugation at 8000 *g* for 10 min, resuspended in 50 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole (pH 8.0), then lysed by sonication on ice, and clarified by centrifugation at 10,000 *g* for 30 min. The supernatant was applied to an affinity Ni^{2+} column (GE Healthcare, Wisconsin, USA). To obtain recombinant proteins, non-specifically bound proteins were removed with 50 mM Tris–HCl, 500 mM NaCl, and 80 mM imidazole (pH 8.0). Recombinant MAT proteins were eluted with 50 mM Tris–HCl, 500 mM NaCl, and 250 mM imidazole (pH 8.0), and then were analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE).

CobB was purified with a maltose binding protein (MBP) tag. Recombinant strains *E. coli* BL21 (DE3)/pMAL-c2X-*cobB* were induced at 37°C for 4 h with 0.5 mM IPTG. Bacterial cells were harvested and resuspended in binding buffer [50 mM Tris-HCl, 500 mM NaCl (pH 8.0)]. Cell pellets were disrupted by sonication and clarified by centrifugation as described above. Supernatants were mixed with an amylose resin column (New England Biolabs, Ipswich, USA). The mixture was washed with 10 volumes of binding buffer to remove non-specifically bound proteins. Recombinant CobB proteins were released with MBP-bind buffer containing 10 mM altose, and then analyzed using SDS-PAGE (**Supplementary Fig. S1**). All of the purified proteins were dialyzed against 50 mM Tris–HCl (pH 8.0) at 4°C. Protein concentrations were measured using Modified Brandford Protein Assay Kit (Sangon Biotech, Shanghai, China).

In vitro auto-acetylation and CobB-mediated deacetylation of MAT

Purified recombinant MAT was incubated with different concentrations of Ac-CoA (Sigma Aldrich, St Louis, USA) in 50 mM Tris–HCl at 37°C for different durations to obtain Ac-MAT. After incubation, all samples were dialyzed against 50 mM Tris–HCl (pH 8.0) to separate Ac-MAT from the mixture. Acetylation levels of Ac-MAT

were determined by western blot analysis and mass spectrometry. CobB-mediated deacetylation of Ac-MAT was performed in the reaction buffer containing 50 mM Tris–HCl (pH 8.5), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 400 μM NAD^+, in a total volume of 30 μl at 25°C overnight. After incubation, samples were divided into three portions: the first portion was used for SDS-PAGE and western blot analysis, the second portion was prepared for enzymatic activity, and the third portion was analyzed using mass spectrometry.

Western blot analysis

For western blot analysis, protein samples were separated by 10% SDS-PAGE and immediately transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany), using BioRad SD device (BioRad Laboratories, Hercules, USA) at 15 V for 30 min. The membrane was blocked at 37°C for 2 h or at 4°C overnight in TBST [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20] with 5% non-fat dry milk. After being washed with TBST for three times, the membrane was incubated with primary rabbit anti-acetylated-lysine polyclonal antibody (1:1000, diluted in TBST/0.5% non-fat dry milk) at 37°C for 2 h. After that, the membrane was washed with TBST three times, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:2000) at 37°C for 1 h. The signal was detected by using chromogenic substrates nitroblue tetrazolium-5-bromo-4-chloro-3′-indolylphosphate (NBT-BCIP) according to the manufacturer's instructions. The antibodies used were all purchased from Cell Signaling Technology (Beverly, USA) unless otherwise indicated.

In vitro enzymatic assay

Enzymatic activity of MAT was measured as previously described [41] with some modification. The assay mixture contained 100 mM Tris–HCl (pH 7.5), 150 mM KCl, 20 mM $MgCl_2$, 1 mM ATP, 0.5 mM methionine, and 5 μl purified recombinant MAT, in a total volume of 300 μl. Reactions were carried out at 37°C for 30 min in triplicate and terminated with the addition of 50 mM EDTA. Enzymatic activity of MAT was assayed by measuring the production of inorganic phosphate (Pi) [42] according to Ames and Dubin [43]. After termination of the enzymatic reaction, 700 μl of freshly prepared ascorbic-molybdate mixture was added, and the mixture was incubated at 45°C for 20 min. After that, absorbance at 820 nm was measured by using the Synergy H1 Multi-Mode Reader (BioTek, Winooski, USA).

Mass spectrometry analysis

To identify the acetylation sites, acetylated and deacetylated MAT were resolved by SDS-PAGE, and stained with Coomassie bright blue R250. Protein spots were cut from the middle of the lanes, and washed with water and stored at −20°C. Samples were sent to Shanghai Applied Protein Technology Company (Shanghai, China) for LC-MS/MS analysis. Protein digestion was performed according to the FASP procedure described by Wisniewski *et al.* [44]. Experiments were carried out on a Q Exactive mass spectrometer which was coupled to Easy nLc (Thermo Fisher, Waltham, USA). The peptide mixture was loaded onto a C18-reversed phase column (Thermo Scientific EASY column, 10 cm long, 75 μm inner diameter, 3 μm resin). For protein identification, the following experiment conditions were used. Enzyme = Trypsin; Max missed cleavages = 2; Fixed modifications: carbamidomethyl (C); Dynamical modifications: oxidation (M), acetyl (K) (protein N-term).

Results

Purification, acetylation, and deacetylation of *E. coli* MAT *in vitro*

E. coli MAT was purified with His-tagged by Ni^{2+} chromatography as described in Materials and Methods section. SDS-PAGE analysis showed that a 42-kDa peptide was successfully expressed and purified, which is consistent with the molecular weight of the anticipated recombinant MAT protein (**Supplementary Fig. S2**). It was reported that CheY could auto-phosphorylate using AcP as the phosphoryl donor [45,46]. To enhance the abundance of MAT acetylation and avoid possible phosphorylation, auto-acetylation was performed using Ac-CoA as the acetyl donor. The efficacy of Ac-CoA for MAT auto-acetylation was examined under various conditions: different durations of treatment and different concentrations of Ac-CoA. Our data suggested that the level of MAT auto-acetylation is dependent on both the length of incubation and the dose of Ac-CoA, as shown in **Fig. 1**. When MAT was incubated with 0.5 mM Ac-CoA for 6 h, a prominent increment of acetylation level could be observed, which was then used for acquiring Ac-MAT. We also tried YfiQ which is the only known acetyltransferase in *E. coli*, but it could not acetylate MAT. For the *in vitro* deacetylation assay, Ac-MAT was incubated with purified recombinant CobB in the presence or absence of NAD^+. As shown in **Fig. 2**, in the presence of NAD^+, CobB could deacetylate Ac-MAT very efficiently. These results confirmed that the acetylation of MAT can be reversed by CobB *in vitro*.

Identification of acetylated lysine residues of *E. coli* MAT

To identify the acetylation sites, purified MAT, auto-acetylated MAT, and CobB-deacetylated MAT were prepared and analyzed using LC-MS [47]. Our results showed that in purified MAT, lysine 18, 37, 98, 209, 222, 246, 266, 270, 370, and 373 were identified to be acetylated. In the auto-acetylated MAT, besides the above lysine residues, lysine 3, 154, 166, 284 were also identified to be acetylated. However, after CobB treatment, no acetylation could be identified on lysine 3, 18, 37, 98, 154, 166, 209, 222, or 270. For instance, as shown in **Fig. 3**, peptide IADQISDAVLDAILEQDPK (37)AR from the acetylated MAT had a molecular mass of 416.26 (**Fig. 3A**), which is 42 Da more than the corresponding peptide of the deacetylated MAT (374.25) (**Fig. 3B**). The peptide NGTLP WLRPDAK(166)SQVTFQYDDGK from Ac-MAT had a molecular mass of 717.36 (**Fig. 3C**), which is 42 Da more than the corresponding peptide in the deacetylated MAT (675.36) (**Fig. 3D**). More detailed data for acetylation analysis of other peptides are included in **Supplementary Fig. S3–8**.

Roles of lysine residues in the activity of MAT

To investigate the roles of acetylated lysine residues in MAT enzymatic activity, site-directed mutagenesis was performed for those lysine residues by substitution with arginine or glutamine, which were genetic mimics for constitutively unacetylated lysine and constitutively acetylated lysine [48]. All mutant proteins were purified as described in Materials and Methods section. However, for the mutants of K18R, K18Q, K166R, K270Q, and K284R, no

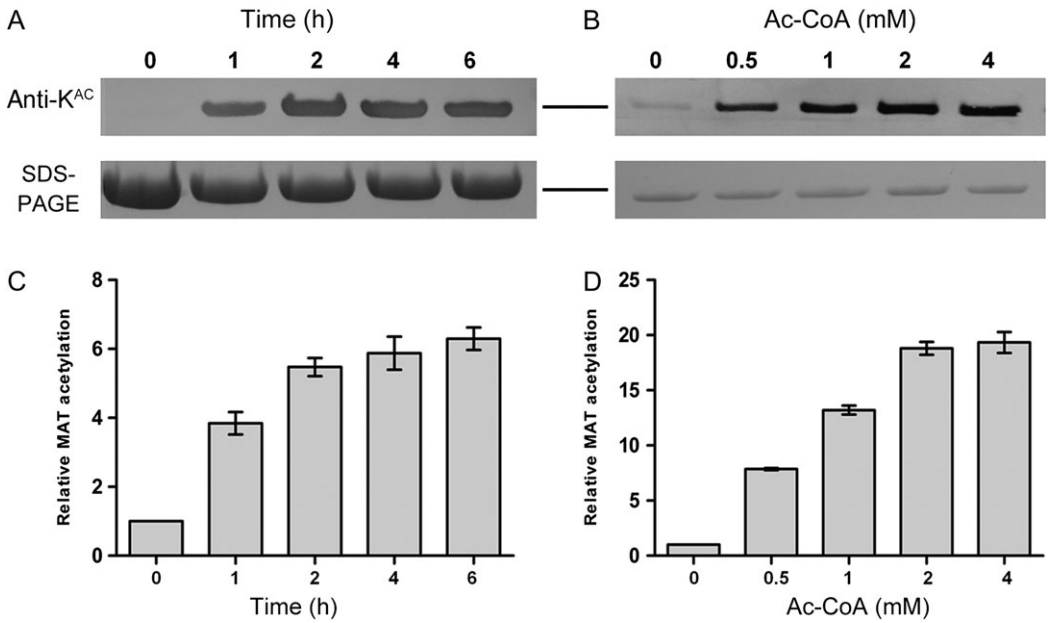

Figure 1. *In vitro* auto-acetylation of *E. coli* MAT (A) The concentration of Ac-CoA was fixed at 0.5 mM, and incubation times were 0, 1, 2, 4, and 6 h, respectively, as indicated. (B) The reaction time was fixed at 6 h and the concentrations of Ac-CoA added were 0, 0.5, 1, 2, and 4 mM, respectively, as indicated. (A, B) Top panel, western blot analysis using an acetyl-lysine-specific antibody. (A, B) Lower panel, SDS-PAGE analysis of MAT. (C, D) Average levels of MAT acetylation were quantified from three independent experiments (mean ± SD) using Gel-pro image analysis software, and normalized to the value obtained in the absence of Ac-CoA [the top panel, first lane in (A) and (B)].

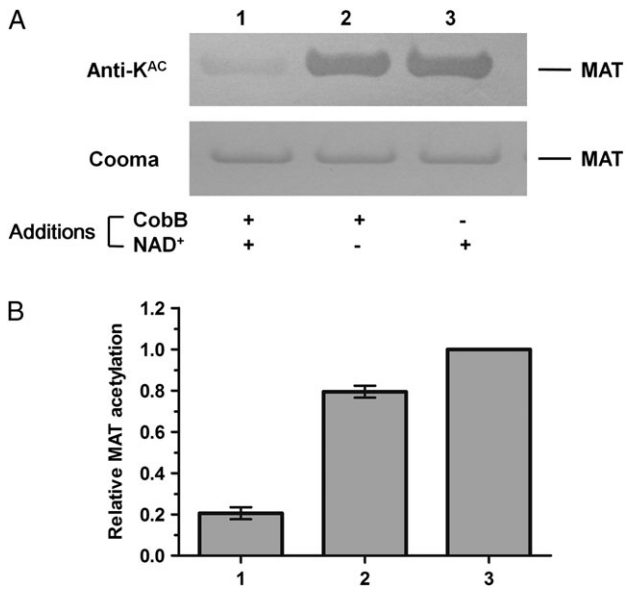

Figure 2. CobB deacetylates MAT *in vitro* Acetylation levels of MAT were determined by an acetyl-lysine specific antibody. (A) Top panel, western blot analysis with an acetyl-lysine-specific antibody; lower panel, SDS-PAGE analysis of MAT by Coomassie bright blue staining. (B) Average levels of MAT acetylation were quantified from three independent experiments (mean ± SD) using Gel-pro image analysis software, and normalized to the value obtained in the absence of CobB.

soluble proteins could be obtained (**Supplementary Fig. S9**). After purification, enzymatic activities of all mutant proteins were determined, and the results showed that mutations of residue Lys37 (K37R and K37Q), Lys209 (K209R and K209Q), Lys222 (K222R and K222Q), Lys370 (K370R and K370Q), or Lys373 (K373R and K373Q) had no effects on the tripolyphosphatase activity of MAT. However, mutations of residue Lys166 (K166Q), Lys246 (K246R and K246Q), Lys266 (K266R and K266Q), and Lys270 (K270R) led to dramatic decreases of the tripolyphosphatase activity. In addition, mutations of K3R, K3Q, K154R, K154Q, and K284Q also led to significant decreases of the tripolyphosphatase activity. Even though substitution of K98 with Q led to a dramatic decrease of the tripolyphosphatase activity, substitution of K98 with R only resulted in a slight decrease of the tripolyphosphatase activity (**Fig. 4**).

Effect of acetylation on MAT enzymatic activity *in vitro*

To further investigate the effects of lysine acetylation on the enzymatic activity of MAT, relative activities of MAT with different levels of acetylation were measured. The specific activity of MAT was calculated according to previous studies [42,43,49]. The definition of one unit of enzyme activity was defined as the amount of enzyme required for producing 1 μM SAM [49]. According to Ames and Dubin [43], 0.01 μM of phosphate produces a change in optical density of 0.240 at 820 nm. We measured the amount of phosphate produced by MAT and converted a change in optical density of 0.240 into 0.01 unit of enzyme activity [42]. After being acetylated by Ac-CoA, the tripolyphosphatase activity of MAT decreased remarkably (**Fig. 5A**). However, deacetylation by CobB restored the tripolyphosphatase activity. These results suggested that acetylation regulates the activity of MAT mediated by CobB *in vitro* (**Fig. 5B**).

Discussion

Though lysine acetylation has been identified in MAT from various species [20,40,50–55], the biological consequence of this

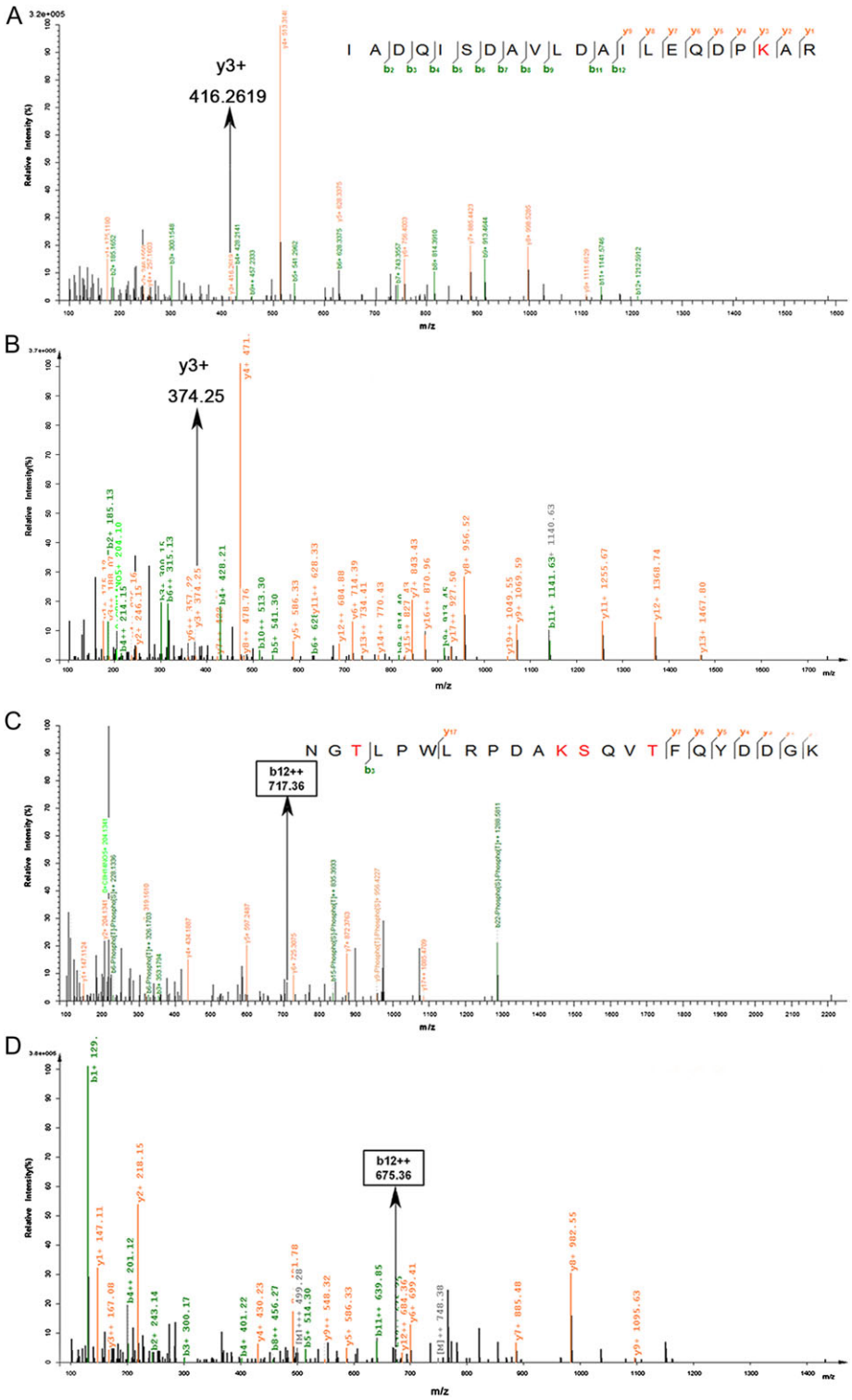

Figure 3. LC-MS/MS analysis confirms that lysine 37 and 166 are acetylated in acetylated MAT and can be deacetylated by CobB (A) Tryptic digestion of acetylated MAT with Lys37 acetylated. The y3+ ion has a mass which is 42 Da greater than that of the corresponding ion in the CobB-treated sample in (B). (C) Tryptic digestion of acetylated MAT with Lys166 acetylated. The b12++ ion has a mass which is 42 Da greater than that of the corresponding ion in the CobB-treated sample in (D). (B, D) Tryptic digestion of CobB deacetylated MAT.

modification still remains unclear. In this work, we found reversible lysine acetylation regulates the activity of *E. coli* MAT *in vitro*.

Previously, six lysine residues of *E. coli* MAT (K3, K246, K266, K284, K370, and K373) have been identified to be acetylated [39,40]. To further identify the acetylated lysine residues in *E. coli* MAT, the protein was over-expressed, purified, and subjected to LC-MS/MS analysis. Compared with previous reports, our data showed that acetylation was not found on K3 and K284

in this work, but 6 more lysine residues were identified to be acetylated (K18, K37, K98, K209, K222, K270). Thus, up to now, among the 20 lysine residues in *E. coli* MAT, 12 have been identified to be acetylated (K3, K18, K37, K98, K209, K222, K246, K266, K270, K284, K370, and K373). To get more insight into the biological meaning of lysine acetylation, protein sequences of MAT from different species were compared, and the data suggest that most of the acetylated lysine residues are conserved (**Fig. 6**), implying that they may play important roles in the enzymatic activity of MAT.

As the only known enzyme which catalyzes the biosynthesis of SAM, the crystal structure of *E. coli* MAT has been solved as the apoenzyme [56–60]. Of the acetylated lysine residues in MAT, three levels of effects were observed with respect to tripolyphosphatase activity brought about by the mutations: low (l), medium (m), and high (h) (**Table 2**). Sequence conservation and structural analysis indicate that the l-effect residues are located on the surface of MAT and are not conserved (**Fig. 6**). The m-effect residues are also exposed, but they formed side chain interactions with neighboring residues. Based on the structure of *E. coli* MAT, we can see that four lysine residues reside in the active sites of the enzyme: Lys166, 246, 266, and 270 [60]. Subsequently, site-directed mutagenesis had been performed to elucidate roles of these four residues on MAT enzymatic activity. Lys166 interacts with one of the phosphoryl groups of PPi through hydrogen bonds, whereas Lys246 and Lys266 form hydrogen bonds with the Pi ion [59,60]. The carboxylate group of the substrate Met moiety forms hydrogen bonds with Lys270 [58]. Thus, mutation of any of the four lysine residues results in a sharp decrease in the enzymatic activity of MAT (h-effect), which was confirmed by previous findings and our data (**Fig. 4**). These results showed that these lysine residues play important roles in the enzymatic activity of MAT.

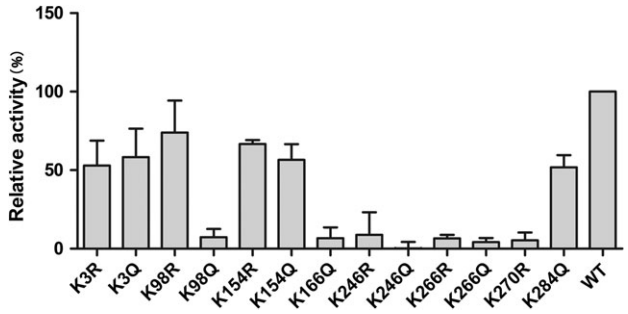

Figure 4. Tripolyphosphatase activities of wild-type and mutant MAT enzymes MAT mutants were expressed in parallel with wild-type (WT) MAT. Data are means ± SD from three independent experiments.

Since acetylation of *E. coli* MAT has been confirmed, we wonder whether MAT acetylation is reversible. So far, the best characterized protein deacetylase of *E. coli* is CobB. So deacetylation of Ac-MAT by CobB was tested *in vitro*, and the results clearly showed that acetylation of *E. coli* MAT could be reversed by CobB (**Fig. 2**). At the same time, data from LC-MS/MS analysis show that no acetylation can be detected on lysine residues 3, 18, 37, 98, 154, 166, 209, 222, and 270 after deacetylation by CobB. These results suggest that acetylation of *E. coli* MAT is reversible *in vitro*. In addition, we noticed that even after long periods of CobB treatment, deacetylation of Ac-MAT was still incomplete, as shown by western blot analysis and LC-MS analysis. A possible explanation may be that CobB is not the only deacetylase that exhibits lysine deacetylase (KDAC) activity [61].

Previous studies have focused on the structural, functional, and regulatory properties of MATs from different sources [31]. Recently, it has been reported that acetylation of mammal MAT IIα protein promotes its ubiquitylation and proteasomal degradation, and inhibits tumor cell proliferation [38]. However, few studies have been carried out on how PTM regulates the activity of *E. coli* MAT. As we proved that MAT undergoes reversible acetylation *in vitro*, we speculate that acetylation may affect the activity of MAT *in vitro*. And our data did show that the enzymatic activity of MAT had a significant decrease when it was acetylated. However, this decrease can be reversed by CobB-catalyzed deacetylation (**Fig. 5**).

The effects of reversible lysine acetylation on the enzymatic activity of MAT may be explained by the following reasons. First, the acetyl groups on these lysine residues may change the structure and/or the combination between enzyme and/or substrates/products. Many acetylated lysine residues identified in this study were reported to be involved in the functional motifs of MAT. For

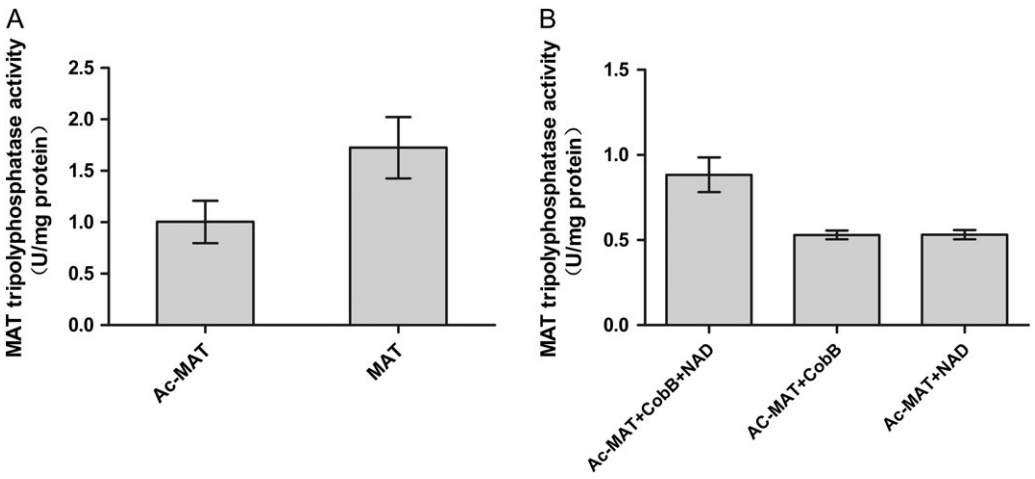

Figure 5. Effects of reversible acetylation on tripolyphosphatase activity of MAT *in vitro* (A) Tripolyphosphatase activity of Ac-MAT was compared with that of MAT. (B) Ac-MAT was deacetylated in presence/absence of CobB and/or NAD^+. Data are means ± SD from three independent experiments.

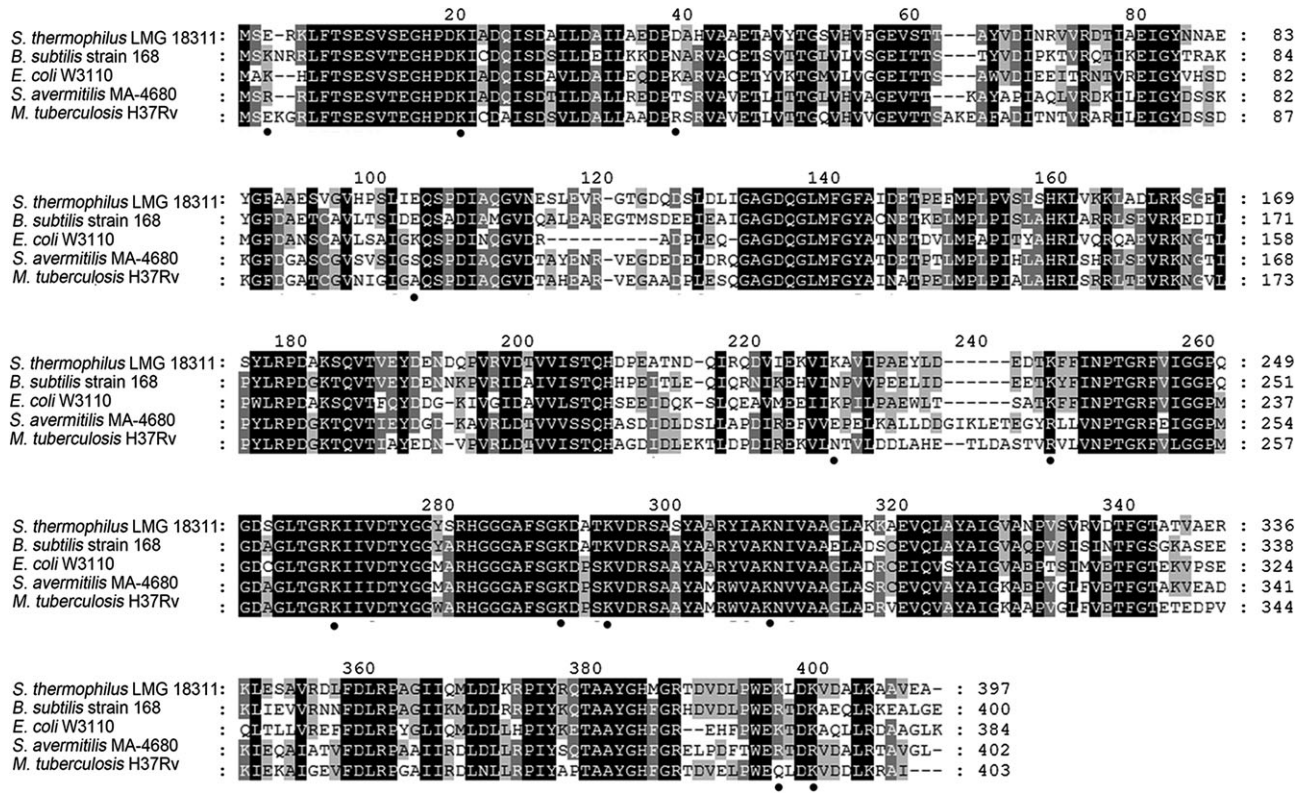

Figure 6. Multiple alignments of MAT from different origins Sequences were aligned using CLUSTAL X. Alignments of MAT from *E. coli* W3110, *Mycobacterium tuberculosis* H37Rv, *Bacillus subtilis* subsp. *subtilis* str. 168, *Streptococcus thermophilus* LMG 18311, and *Streptomyces avermitilis* MA-4680 were performed. Conserved residues were highlighted in black. The lysine residues undergo acetylation *in vivo* were indicated by black dot (●).

Table 2. The feature of acetylated lysine residues in MAT

Residue	Insoluble[a]	Activity[b]	Structural features
K3		R/Q (m)	Exposed, salt bridge w/D174
K18	R/Q		Buried
K37		R/Q (l)	Exposed
K98		Q (h) R (m)	Intermolecular, salt bridge
K154		R/Q (m)	Exposed, salt bridge w/E13
K166	R	Q (h)	Active site
K209		R/Q (l)	Exposed
K222		R/Q (l)	Exposed
K246		R/Q (h)	Active site
K266		R/Q (h)	Active site
K270	Q	R (h)	Active site
K284	R	Q (m)	Partially exposed, hydrophobic w/113L, 279R, and 370T, and salt bridge w/E364 and E369
K370		R/Q (l)	Exposed
K373		R/Q (l)	Exposed

[a]Mutation which produces insoluble protein.

[b]Three levels of inhibitory effect on tripolyphosphatase activity brought about by mutations: low (l), medium (m), and high (h).

instance, the carboxylate group of the substrate Met moiety forms hydrogen bonds with Lys270 [58]. Lys166 interacts with one of phosphoryl groups of PPi by hydrogen bonds [59,60]. When acetyl group was transferred to ε-amine, the positive charge in the ε-amino group of lysine can be eliminated and the hydrogen bonds may be destroyed, and thus the structure and/or conformation of MAT may be changed (**Fig. 7A**). Residue K98 forms inter-molecular salt bridges in the dimer–dimer interface with residues D64 and E67 (**Fig. 7B**), and mutations at this residue showed different effect levels. Mutations to arginine (K98R) and glutamine (K98Q) had m-effect and h-effect (**Table 2**), respectively. Because the arginine side chain with positively charged guanidinium may still be capable of forming intermolecular salt-bridges, while the glutamine may abolish such interactions. Acetylation of K98 may affect the interaction between two dimers, and thus affects the activities of MAT. Therefore, changes of acetylation level can affect the activity of MAT.

MAT is the key enzyme catalyzing the synthesis of SAM, which is recognized as the major-methyl donor reagent in metabolism. The procedure of methyl transfer from SAM to nucleic acids has important effects on DNA replication and transcription, as well as on RNA function [62]. In *E. coli*, reduction in SAM pool size caused by the expression of bacteriophage T3 SAM hydrolase results in increased hemimethylated or unmethylated GATC sites in the genome [35]. In addition, the homoserine lactone ring of LuxI-family autoinducers which are known as quorum-sensing molecules in *E. coli* is derived from SAM [63]. In addition, SAM is also used as a source of ribosyl groups in the penultimate step of the biosynthetic pathway of queuosine, a hypermodified tRNA nucleoside [25]. As mentioned above, our data show that MAT can be acetylated non-enzymatically by Ac-CoA, and that CobB can deacetylate Ac-MAT *in vitro*. Furthermore, we proved that reversible acetylation might

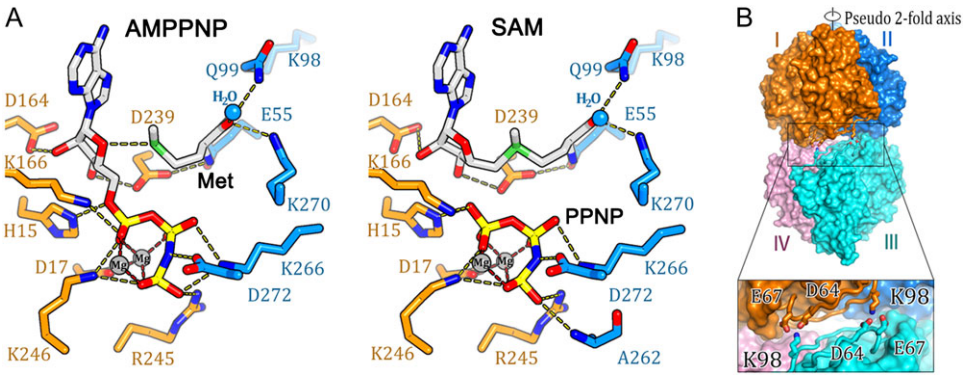

Figure 7. Structural analysis of the lysine acetylation-related inhibitory effect to the MAT tripolyphosphatase activity (A) Active site view of MAT substrate (left) and product (right) complexes. The active site is formed between two MAT molecules (I in orange and II in blue). Residues K166, K246, K266, and K270 are involved in substrate- and product-binding, while residue K98 points away from active site. Magnesium ions are shown in spheres. Dashed lines indicate hydrogen bonding and salt bridge (both in yellow), or coordination interactions (in red). (B) Overview of the MAT tetramer formed in the crystal lattice. Residue K98 forms inter-molecular salt bridges in the dimer–dimer interface with residues D64 and E67. The four monomers are colored in orange, blue, cyan, and pink, respectively. A mixture of surface, cartoon, and stick representation are used for a better view of global tetramer arrangement and the interaction details involving K98. K98 participates in four sets of interactions. Among these, two sets are visible from the current view. The other two sets are invisible and correlated with the visible sets through the indicated pseudo 2-fold axis.

modulate the activity of MAT. This reveals a novel mechanism of *E. coli* MAT regulation by acetylation *in vitro*.

Supplementary Data

Supplementary data is available at *ABBS* online.

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