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Original Article

Purification and characterization of the acetyl-CoA synthetase from *Mycobacterium tuberculosis*

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Acetyl-CoA (AcCoA) synthetase (Acs) catalyzes the conversion of acetate into AcCoA, which is involved in many catabolic and anabolic pathways. Although this enzyme has been studied for many years in many organisms, the properties of *Mycobacterium tuberculosis* Acs and the regulation of its activity remain unknown. Here, the putative *acs* gene of *M. tuberculosis* H37Rv (Mt-Acs) was expressed as a fusion protein with 6×His-tag on the C-terminus in *Escherichia coli*. The recombinant Mt-Acs protein was successfully purified and then its enzymatic characteristics were analyzed. The optimal pH and temperature, and the kinetic parameters of Mt-Acs were determined. To investigate whether Mt-Acs is regulated by lysine acetylation as reported for *Salmonella enterica* Acs, its mutant K617R was also generated. Determination of the enzymatic activity suggests that Lys-617 is critical for its function. We further demonstrated that Mt-Acs underwent auto-acetylation with acetate but not with AcCoA as the acetyl donor, which resulted in the decrease of its activity. CoA, the substrate for AcCoA formation, inhibited the auto-acetylation. Furthermore, the silent information regulator (Sir2) of *M. tuberculosis* (Mt-Sir2) could catalyze Mt-Acs deacetylation, which resulted in activation of Acs. These results may provide more insights into the physiological roles of Mt-Acs in *M. tuberculosis* central metabolism.

Keywords acetyl-CoA synthetase; acetate; auto-acetylation; deacetylase; *Mycobacterium tuberculosis*

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Introduction

Adenosine monophosphate (AMP)-forming acetyl-CoA (AcCoA) synthetase (Acs, EC 6.2.1.1) is a key enzyme for conversion of acetate into AcCoA, an essential intermediate at the junction of anabolic and catabolic pathways. The enzyme is a member of the acyl–adenylate forming enzyme superfamily, and is widespread in all three domains of life. In bacteria, Acs is the preferred route of acetate assimilation when the concentration of acetate in the environment is low (≤10 mM). In eukaryotes, Acs is the only route for the activation of acetate into AcCoA [1,2].

This enzyme undergoes a two-step reaction. In the first-half reaction, Acs combines acetate with ATP to form acetyl–adenylate (AcAMP) intermediate (Equation 1). In the second-half reaction, it can then transfer the acetyl group from AcAMP to the sulfhydryl group of CoA, forming the product AcCoA (Equation 2) [3–6].

$$\mathrm{E} + \mathrm{acetate} + \mathrm{ATP} \leftrightarrow \mathrm{EAcAMP} + \mathrm{PPi} \quad (1)$$

$$\mathrm{EAcAMP} + \mathrm{HSCoA} \leftrightarrow \mathrm{E} + \mathrm{AcCoA} + \mathrm{AMP} \quad (2)$$

Furthermore, recent evidence supported the hypothesis that acetylation broadly impacts bacterial physiology [7–10]. However, most information about acetylation in bacteria comes from the study of the Acs protein. Reversible acetylation of a single lysine residue regulates Acs activity [10]. In *Salmonella enterica*, Acs activity is modulated by acetylation/deacetylation of residue Lys-609, which is critical to the synthesis of the AcAMP intermediate from acetate and ATP [11,12]. The protein acetyltransferase (Pat) uses AcCoA as a substrate to acetylate Acs, which deactivates it [13]. Reactivation of the acetylated Acs (AcAcs) requires the silent information regulator (Sir2). It is a member of the sirtuin family of deacetylases homologous to the CobB enzyme of *S. enterica*, which removes acetyl group from AcAcs [11]. Moreover, the Acs enzyme of *Escherichia coli* was also found to undergo auto-acetylation in the presence

of acetate and ATP [14], but the mechanism of this kind of auto-acetylation still remains obscure.

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the most devastating global diseases [15]. Long-term antibiotic therapy has led to the emergence and wide spread of multi-drug-resistant *M. tuberculosis* strains [16]. To shorten the duration of chemotherapy, new drugs targeting persistent *M. tuberculosis* are urgently needed [17]. However, our knowledge of bacteria metabolism throughout the course of infection remains rudimentary. AcCoA is the substrate for the glyoxylate shunt, which is shown to be involved in *M. tuberculosis* persistence and up-regulated during *M. tuberculosis* growth arrest [18,19]. Moreover, previous researches showed that Acs of *E. coli* might control the expression and/or activity of the glyoxylate bypass [2,20]. Therefore, *M. tuberculosis* Acs may play a role in *M. tuberculosis* persistence. However, characterization of *M. tuberculosis* Acs has not been reported. In this work, we successfully expressed the recombinant *M. tuberculosis* H37Rv Acs with a 6×His-tag on the C-terminus (Mt-Acs) in *E. coli*. The recombinant Acs protein was purified by nickel–nitrilotriacetic acid (Ni–NTA) His-binding resin affinity chromatography, and then its enzymatic properties and kinetic parameters were determined. In order to investigate the active site of Mt-Acs, the mutant K617R was also prepared. To determine the mechanism of Mt-Acs activity regulation, western blot, and enzyme-activity assay were performed.

Materials and Methods

Bacterial strains, growth conditions, and plasmids

E. coli DH5α was used as the host for cloning. The *M. tuberculosis* Acs protein was expressed using *E. coli* BL21 (λDE3) strain, while Sir2 protein was expressed in *E. coli* strain AD494 (λDE3) with the genotype $\triangle ara^{-}$ *leu7697* Δ*lacX74* Δ*phoAPvuII phoR* Δ*malF3 F′* [lac^{+} *(lacI*^{q}*)* pro] *trx*B::kan (DE3). All strains were obtained from our laboratory stock. Plasmids pET20b and pET32a (Novagen, Darmstadt, Germany) were used to construct vectors for the expression of *M. tuberculosis* Acs and Sir2 proteins, respectively. The medium used was Luria-Bertani (LB) medium. Ampicillin (Ap) (100 μg/ml) and kanamycin (50 μg/ml) were added if needed. All chemicals were purchased from Sigma (St Louis, USA) unless otherwise specified.

Construction of protein expression vectors

The *rv3667* (*acs*) gene was amplified from *M. tuberculosis* H37Rv genomic DNA (obtained from Wuhan Tuberculosis Hospital, Wuhan, China) by *LA Taq* polymerase (Takara, Dalian, China) with GC buffer. The nucleotide sequence of forward and reverse primers for the reaction were 5′-CAGGCATATG(*Nde*I)AGTGAGTCCACCCCCGAAGT-3′ and 5′-ATATAAGCTT(*Hind*III)CTTGGCGGCCCGGATCG-3′, respectively. The polymerase chain reaction (PCR) product (1970 bp) was cloned into the *Nde*I and *Hind*III sites of pET20b to generate pET20b-*acs*. The recombinant expression vector was confirmed by restriction enzyme digestion and DNA sequencing, and then introduced into *E. coli* BL21 (λDE3) by $CaCl_2$ transformation and the antibiotic-resistant transformants were selected for expression experiment. The *rv1151c* (*sir2*) gene was ligated to pET32a and the resulting plasmid pET32a-*sir2* was transformed into *E. coli* AD494 (λDE3) for protein expression.

Site-directed mutagenesis

In order to investigate the active site of Mt-Acs, site-directed mutation was introduced into the selected site in the *acs* gene by overlap PCR [21]. The fragment was ligated into pET20b, and was completely sequenced to confirm the presence of the site-directed mutation. Mutagenic primers were given below with the mutated base triplets underlined: 5′-GACTCGTAGCGGCAGAATCATGCGTCGACT-3′ (sense) and 5′-AGTCGACGCATGATTCTGCCGCTACGAGT-3′ (antisense).

Protein expression and purification

E. coli BL21 (λDE3)/pET20b-*acs* was grown in 5 ml LB medium, and then the overnight culture was transferred into 500 ml fresh LB medium containing Ap at 37°C in shaking flasks to optical density at 600 nm of 0.4. The temperature was adjusted to 18°C and the expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to 0.2 mM. The cells were grown for an additional 12 h at 18°C, harvested by centrifugation, and then resuspended in binding buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 5 mM imidazole]. The cell suspension was then sonicated on ice at the intensity of 3 s burst at 200 W with a 5 s cooling period between each burst with an ultrasonic cell disruptor (VCX750; Ningbo Scientz Biotechnology, Ningbo, China). The lysate was centrifuged (12,000 *g* for 30 min at 4°C) and supernatant was applied to an affinity Ni^{2+} column pre-equilibrated with the binding buffer. According to the manufacturer's protocol (Novagen), the column was washed with binding buffer, followed by washing buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 80 mM imidazole]. The histidine-tagged protein was eluted with elution buffer [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 250 mM imidazole]. The purity of eluted protein was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide-gel electrophoresis), and the peak fractions containing Acs were pooled and concentrated by ultrafiltration using a 30,000 molecular weight cut-off concentrator (Millipore, Billerica, USA) in storing buffer [50 mM Tris-HCl (pH

8.0)]. The Mt-Sir2 and the mutant of Mt-Acs proteins were expressed and purified as described above. Protein concentrations were measured by the Bradford Protein Assay Kit (Beyotime, Nantong, China).

Enzyme activity assay

Enzymatic activity of Mt-Acs was determined by monitoring AcCoA formation from acetate, ATP and CoA by the hydroxamate reaction [22,23], in which activated acyl groups are converted to an acyl-hydroxamate and subsequently to a ferric hydroxamate complex that can be detected spectrophotometrically at 540 nm. Reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 600 mM hydroxylamine-HCl (pre-neutralized with KOH), 10 mM $MgCl_2$, and 2 mM DTT, with varied concentrations of acyl substrate, CoA, and ATP. Reactions were preincubated at 37°C for 5 min before the addition of purified Mt-Acs (5 μM), and terminated by addition of an equal volume of stop solution [2.5% (w/v) $FeCl_3$ in 2 M HCl, 10% (w/v) trichloroacetic acid]. The end products were centrifuged for 1 min in an Eppendorf tube to remove turbidity, and the optical density (OD) at 540 nm was measured. Samples without Mt-Acs served as a blank, and formation of acetyl-hydroxamate by acetyl-phosphate (Sigma) served as the standard. Specific activity was given as μmole min^{-1} mg^{-1}. No Acs activity was detectable in the absence of CoA. For the determination of apparent kinetic parameters, the concentration of one substrate (acyl substrate, CoA, and ATP) was varied and the other two substrates were kept constant at saturating concentrations as follows (100 mM potassium acetate, 30 mM $MgCl_2$, 30 mM ATP, and 1 mM CoA). The apparent steady-state kinetic parameters and their standard errors were determined by non-linear regression to fit the data to the Michaelis–Menten equation.

Auto-acetylation assay

Purified Mt-Acs protein (10 μM) was incubated at 37°C for 2 h with potassium acetate (KAc, 10 mM) in the presence of 10 mM $MgCl_2$, 10 mM ATP, and 50 mM Tris-HCl (pH 8.0) [14]. The reaction was terminated by the addition of SDS sample buffer, and then separated by 12% SDS-PAGE. The acetylation level was analyzed by western blot using specific anti-acetylated-lysine antibody (Cell Signaling Technology, Danvers, USA).

Mt-Sir2-mediated deacetylation of Mt-Acs

After the Mt-Acs auto-acetylation mixture was incubated at 37°C for 6 h, AcAcs was separated from the reaction mixture by ultrafiltration. To examine whether auto-acetylated AcAcs can be deacetylated, 50 mM Tris-HCl (pH 8.5), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 400 μM NAD^+, and 10 μM Mt-Sir2 protein were added to the separated AcAcs protein (10 μM). The mixture was incubated at 25°C for 10 h. The samples were each divided into two portions: one portion was resolved by SDS-PAGE and analyzed by western blot, and the other was used for measurement of the Acs activity.

Western blot analysis

For western blot analysis, the samples were separated by SDS-PAGE and then transferred semidry on a BioRad SD device (BioRad Laboratories, Hercules, USA) for 30 min at 15 V to a poly(vinylidene difluoride) membrane. The membrane was blocked overnight at 4°C in 1×TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk (NFDM). Primary rabbit anti-acetylated-lysine polyclonal antibody diluted 1:1000 in TBST/0.5% NFDM was used. After incubation at 37°C for 2 h, the blot was washed with TBST and incubated with 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit antibody (Boster Bio-Technology, Wuhan, China) for 1 h at 37°C. The color development was achieved by reacting with chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium.

Results

Protein expression and purification

As the (G + C)% of *rv3667* (*acs*) gene is almost 65%, *LA Taq* polymerase (Takara) with GC buffer was selected to amplify the gene from *M. tuberculosis* H37Rv genomic. Then the *acs* gene was successfully cloned into the expression vector pET20b and the recombinant Mt-Acs with a 6×His-tag at the C-terminus was expressed in *E. coli*. In order to obtain maximum soluble Mt-Acs protein, different expression conditions were tested. These included the use of different growth media and different temperatures and times of induction. The highest percentage of soluble protein was obtained when the stains were grown to OD_{600} = 0.4, and then induced with 0.2 mM IPTG at 18°C for 12 h. The recombinant Mt-Sir2 protein was expressed with a 6×His-tag and a Trx-Tag at the N-terminus in *E. coli*.

Ni–NTA His-binding resin affinity chromatography was used to purify the soluble recombinant Mt-Acs. After washing with washing buffer containing 80 mM imidazole, the protein was eluted with elution buffer containing 250 mM imidazole. SDS-PAGE results (**Fig. 1**) showed that a Mt-Acs protein fraction was eluted with 250 mM imidazole, and a pure band migrated slightly slower than the 66.2 kDa protein standard, and was consistent with the estimated mass of Mt-Acs (71.4 kDa). Then the protein was concentrated and imidazole was fully removed by ultrafiltration with 50 mM Tris-HCl (pH 8.0). Finally, approximately 2 mg of the Mt-Acs protein was obtained

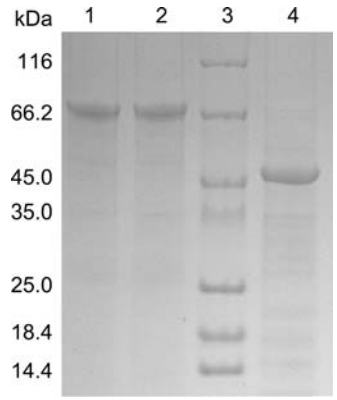

Figure 1 SDS-PAGE analysis of purified recombinant proteins of *M. tuberculosis* Lanes 1 and 2, wild-type Mt-Acs and the mutant K617R, respectively; lane 3, the molecular mass marker; lane 4, Mt-Sir2 protein.

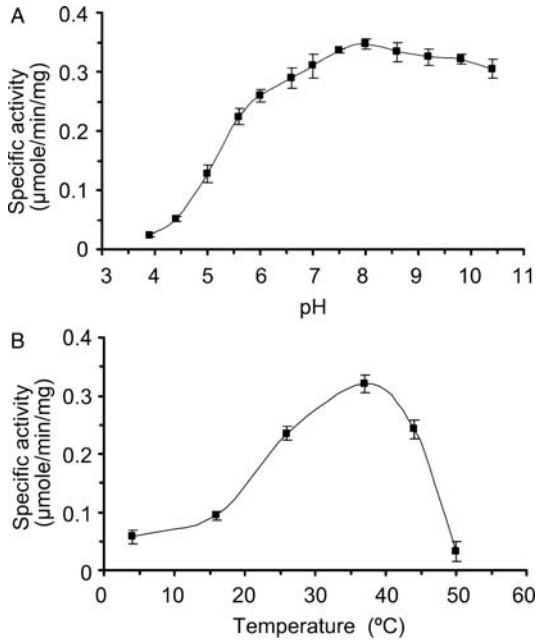

Figure 2 Effect of pH and temperature on Mt-Acs (A) pH profile of Mt-Acs. Acetic acid/sodium acetate (pH 3.6–6.0), disodium hydrogen phosphate/sodium dihydrogen phosphate (pH 6.0–8.0), and glycine/sodium hydrate (pH 8.6–10.4) buffers were used for the measurements, and the buffer concentrations were adjusted to 100 mM. (B) Temperature profile of Mt-Acs. Disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (100 mM, pH 8.0) was used as the solvent. Data were expressed as mean ± SD of three independent experiments.

from 500 ml bacterial culture. To prevent loss of activity, the protein was stored at −80°C in the presence of 10 % (v/v) glycerol. Under such conditions, the activity was fully preserved for at least 2 months. Besides, the purification results of Mt-Sir2 (46 kDa) and the mutant of Mt-Acs K617R proteins were also shown in **Fig. 1**.

Ezymatic activity of Mt-Acs

The specific activity of the Mt-Acs protein was 0.3–0.5 μmole min^{-1} mg^{-1}. This value was close to the specific activity of Acs isolated from the bacterium *Alcaligenes eutrophus* (0.6 μmole min^{-1} mg^{-1}) [24], lower than that isolated from *S. enterica* (3.9–4.7 μmole min^{-1} mg^{-1}) and *Bradyrhizobium japonicum* (16 μmole min^{-1} mg^{-1}) [25,26]. This value was also lower than the specific activities of AMP-forming Acs proteins of eukaryotes (30–60 μmole min^{-1} mg^{-1}) [27,28].

The effects of pH and temperature on enzyme activity were determined. The optimal pH was found to be around pH 8.0 [**Fig. 2(A)**]. The enzyme was stable under neutral and alkaline conditions, while its activity decreased rapidly below pH 6.0. The optimal temperature for the enzyme was 37°C [**Fig. 2(B)**]. Mt-Acs lost its activity rapidly below 25°C or above 45°C.

The metal specificity was also tested using 10 mM of various metal ions. As shown in **Fig. 3**, Mg^{2+}, which was known to be required for the Acs activity [14], could be substituted with Ca^{2+} and Mn^{2+}, whereas Ni^{2+} and Zn^{2+} worked poorly for the enzyme.

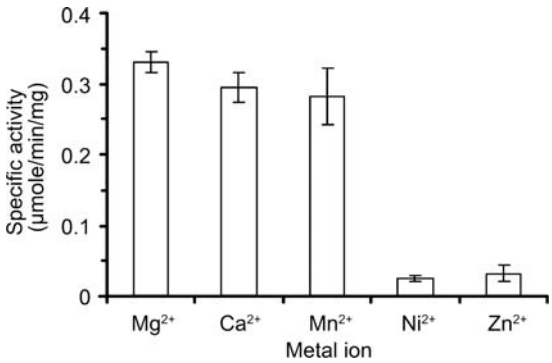

Figure 3 Divalent metal specificity for Mt-Acs Enzyme reactions were performed at 37°C in the presence of 10 mM metal (as chloride salt). Data were expressed as mean ± SD of three independent experiments.

Table 1 Kinetic analysis of Mt-Acs

Substrate	K_m (mM)	k_{cat}(s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Acetate	1.2 ± 0.13	0.3 ± 0.01	0.28 ± 0.03
Propionate	2.1 ± 0.3	0.2 ± 0.01	0.1 ± 0.01
Butyrate	509 ± 68	0.2 ± 0.003	$4 \times 10^{-4} \pm 4.7 \times 10^{-5}$
ATP[a]	5.6 ± 1.08	0.59 ± 0.06	0.11 ± 0.01
CoA[a]	0.35 ± 0.06	0.93 ± 0.12	2.63 ± 0.09

The data are presented as the mean ± SD of triplicate tests.

[a]The values for ATP and CoA were determined using acetate as the substrate.

Kinetic analysis of Mt-Acs

The kinetic parameters of Mt-Acs were determined (**Table 1**). The dependence of the reaction rate on acyl substrates, ATP, and CoA concentrations followed

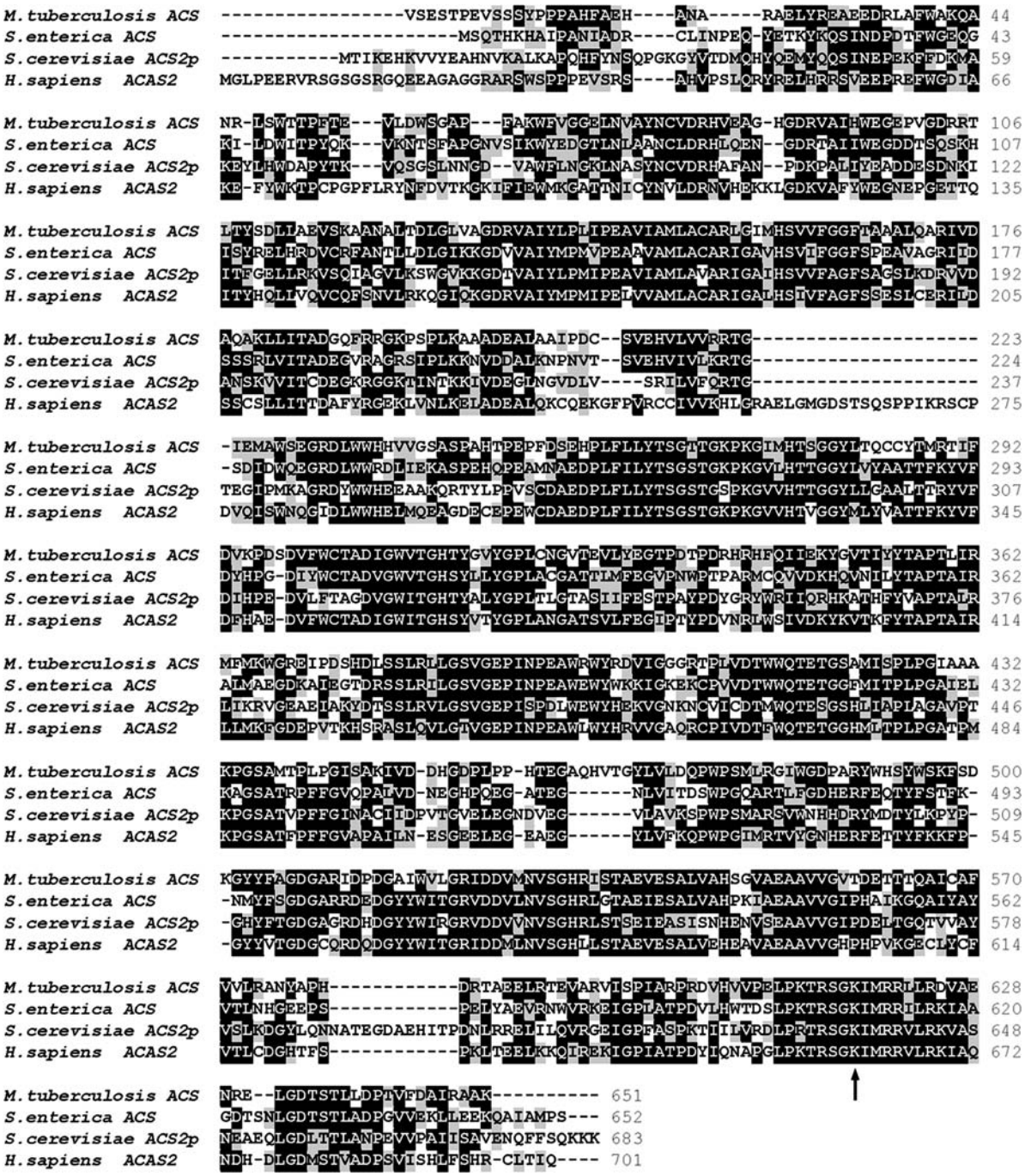

Figure 4 Multiple sequence alignment of acetyl-CoA synthetases from different species The alignment was made using the MEGALIGN program (CLUSTAW). The active-site lysine was marked by the black arrow.

Michaelis–Menten kinetics. Acetate was the preferred substrate for the enzyme with a K_m of 1.2 mM. However, Mt-Acs has only a 2-fold higher affinity and catalytic efficiency (k_{cat}/K_m) with acetate than with propionate. Butyrate was a very poor substrate for Mt-Acs with a K_m of 509 mM and a k_{cat}/K_m 700-fold lower than acetate. K_m values for ATP and CoA were 5.6 and 0.35 mM, respectively, which was higher than other reported Acs [26,29].

Effect of the mutation at residue K617 on enzyme activity

Previous studies suggested that the lysine-609 in the *S. enterica* Acs was a key residue for the adenylation reaction [11]. Based on the conservation of the active-site region of *S. enterica* Acs with *M. tuberculosis* Acs and Acs from other species (**Fig. 4**), we tested whether mutation of Lys-617 would affect the Acs activity of Mt-Acs. The result showed that replacement of Lys-617 with arginine yielded a

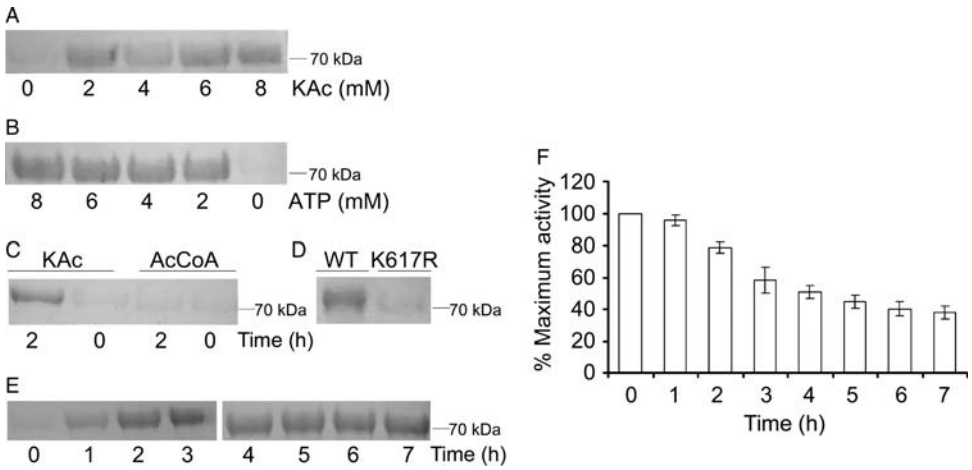

Figure 5 Mt-Acs auto-acetylation The acetylation levels of all proteins were determined by western blot using specific anti-acetyl lysine antibody. (A) Dependence on the presence of KAc. ATP (10 mM) was incubated for 2 h at 37°C with the indicated concentration of KAc. (B) Dependence on the presence of ATP. KAc (10 mM) was incubated for 2 h at 37°C with the indicated concentration of ATP. (C) Effect of AcCoA on Mt-Acs auto-acetylation. The reaction mixture contained KAc (2 mM) or AcCoA (2 mM), and ATP (10 mM). The reaction was carried out at 37°C, and Mt-Acs was separated at indicated time points. (D) Lys-617 is required for Mt-Acs auto-acetylation. Wild-type (WT) Mt-Acs and mutant K617R were used at equal amounts for auto-acetylation assay. (E) Time-dependent auto-acetylation of Mt-Acs. At the indicated time points, aliquots of the reaction mixture were collected and resolved by SDS-PAGE. (F) The enzyme activity of Mt-Acs after auto-acetylation for the indicated time was measured. Activity is described as a percentage of the maximum activity determined for Mt-Acs before auto-acetylation. Data were expressed as mean ± SD of three independent experiments.

complete loss of Mt-Acs activity, indicating that Lys-617 is critical for Mt-Acs function (**Supplementary Figure S1**).

Mt-Acs auto-acetylation

Incubation of purified recombinant Mt-Acs protein with KAc and ATP at 37°C resulted in the formation of an acetyl-Acs derivative. After incubation, the protein was denatured, and the acetylation level was tested using anti-acetyl-lysine antibody (**Fig. 5**). The addition of acetyl groups to Mt-Acs was shown to be dependent on the presence of KAc [**Fig. 5(A)**] and ATP [**Fig. 5(B)**].

Previous observations that AcCoA was the acetyl donor in many auto-acetylation systems [14,30–32] raised the question as to whether AcCoA could also serve as an acetyl donor for Mt-Acs auto-acetylation. To examine this possibility, AcCoA instead of acetate was incubated with Mt-Acs for 2 h. Western blot analysis results demonstrated that no auto-acetylation of Mt-Acs occurred in the presence of AcCoA [**Fig. 5(C)**]. Thus, AcCoA is not responsible for Mt-Acs auto-acetylation.

Since Lys-617 was shown to be critical for Mt-Acs function, the role of Lys-617 on auto-acetylation was also tested. As shown in **Fig. 5(D)**, K617R had nearly no auto-acetylation in comparison with wild-type protein. Therefore, the results showed that Lys-617 was required for auto-acetylation, which was reported to be the site of acetylation.

In order to investigate whether auto-acetylation of Mt-Acs affects its enzyme activity, aliquots were taken at various time points, and then analyzed by western blot and enzymatic activity assays. As shown in **Fig. 5(E)**, the acetylation level of Mt-Acs gradually increased as the incubation time extended. Simultaneously, measurement of the specific activity of Mt-Acs with different levels of acetylation, as described above, indicated that acetylated Mt-Acs had a significantly lower specific activity [**Fig. 5(F)**]. These findings are consistent with the hypothesis that auto-acetylation of Mt-Acs inhibited its enzymatic activity.

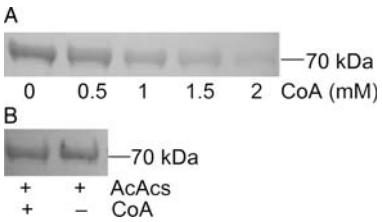

Figure 6 Effect of the CoA on Mt-Acs auto-acetylation The acetylation levels of all proteins were determined by western blot using specific anti-acetyl lysine antibody. (A) CoA at the indicated concentrations was incubated with the auto-acetylation mixture at 37°C for 2 h. (B) CoA at the high concentration (2 mM) was incubated with the separated AcAcs protein. Both reactions, with or without CoA, were incubated at 37°C for 6 h.

Effect of the CoA on Mt-Acs auto-acetylation

Previous studies revealed that Acs firstly catalyzed the formation of AcAMP from acetate and ATP, then transferred the acetyl group from AcAMP to CoA [3,29,33]. Data obtained in this study showed that Mt-Acs also underwent auto-acetylation in the presence of acetate and ATP. To test

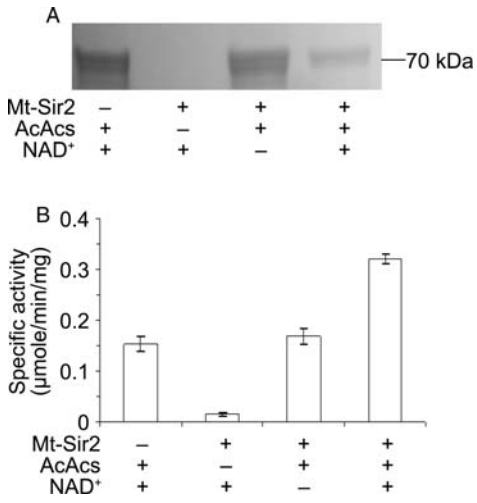

Figure 7 ***In vitro*** **deacetylation of Mt-Acs by Mt-Sir2** (A) The acetylation levels of all proteins were determined by western blot using specific anti-acetyl lysine antibody. (B) Deacetylation of Mt-Acs by Mt-Sir2 activated its acetyl-CoA synthetase activity. Data were expressed as mean ± SD of three independent experiments.

whether CoA might affect auto-acetylation, the auto-acetylation mixture was incubated with increasing concentrations of CoA at 37°C for 2 h, then subjected to SDS-PAGE and analyzed by western blot [14]. The results showed that CoA effectively inhibited auto-acetylation [**Fig. 6(A)**]. However, when CoA was directly incubated with auto-acetylated AcAcs protein, the acetylation level of Mt-Acs was constant despite the presence of CoA [**Fig. 6(B)**]. This suggests that acetylated Mt-Acs cannot transfer its acetyl group to CoA, which supports the conclusion that a covalent bond forms between Mt-Acs and the acetyl group.

Reversibility of Mt-Acs acetylation

To examine whether the acetylated Mt-Acs undergoes deacetylation, the separated AcAcs was incubated with Mt-Sir2 protein and NAD^+. The results showed that Mt-Sir2 reduced acetylation levels of AcAcs, while no reduction of acetylation was observed when Mt-Sir2 or NAD^+ was withdrawn from the reaction [**Fig. 7(A)**]. The results demonstrated that the Mt-Acs acetylation is reversible and Mt-Sir2 is responsible for this process. Measurement of the Acs activity of AcAcs with different treatment, as described above, showed that deacetylation by Mt-Sir2 was necessary for activation of enzymatic activity [**Fig. 7(B)**].

Discussion

Although Acs has been studied extensively, Acs from the human pathogen *M. tuberculosis* has not been characterized [14,24–26,34–38]. In this study, Mt-Acs was successfully cloned, over-expressed, and purified and subjected to biochemical analysis. Our main findings are discussed below.

From the data obtained, we conclude that Mt-Acs synthesizes AcCoA from acetate, ATP, and CoA, although the specific activity of Mt-Acs (0.3–0.5 μmole min^{-1} mg^{-1}) was lower than Acs from most other tested species. Like other family members, the K_m value for acetate observed for Mt-Acs was determined to be 1.2 mM, which is 6 folds higher than that observed for *E. coli* (K_m = 0.2 mM) [36]. Compared with *E. coli* Acs, Mt-Acs has lower affinity for acetate. Meanwhile, Mt-Acs showed poor affinity and catalytic efficiency for ATP and CoA compared with other Acs enzymes [26,29]. These characteristics are consistent with the observation that low concentration of acetate (10 mM) cannot support the growth of *M. tuberculosis* (data not shown). Whereas *E. coli* grew well under low concentration of acetate (2.5 mM), in which Acs has a specific activity of 1.8–4.4 μmole min^{-1} mg^{-1} [14,37].

Besides, Mt-Acs showed only a weak (2-fold) preference for acetate over propionate, which is different from Acs of *E. coli* (5–6-fold) and most other Acs (generally 10–20-fold) [26,29,38]. In fact, the preference of Mt-Acs for acetate resembled Acs from *Archaeoglobus fulgidus* [38]. Very strikingly, Mt-Acs was able to utilize butyrate as substrate, although the K_m was 500 fold higher than that for acetate. Meanwhile, no PrpE homolog in Mt H37Rv could be found by protein BLAST. This indicates that *M. tuberculosis* may use the same enzyme for both acetate and propionate assimilation.

Another fact has to be mentioned is the optimal temperature for the Acs enzyme was 37°C, which is consistent with the optimal growth temperature of *M. tuberculosis* both *in vitro* and *in vivo*.

It has been shown that *E. coli* Acs underwent auto-acetylation in the presence of acetate and ATP [14]. Through western blot analysis, the same mechanism of acetylation was also found in Mt-Acs. Besides, when the protein was over-expressed and purified, it was shown to be already acetylated to a low extent, indicating that Mt-Acs maintains a basal level of acetylation *in vivo* (**Fig. 5**). However, the role of auto-acetylation *in vivo* still needs to be established.

Previous studies demonstrated that auto-acetylation existed in selected acetyltransferase and transcription factors, with AcCoA as the acetyl donor and resulted in the activation of the autoacetylated proteins [14,30,31,39,40]. However, in the case of Mt-Acs, AcCoA could not serve as an acetyl donor for auto-acetylation and was replaced by acetate instead. As expected, auto-acetylation of Mt-Acs regulates its own activity, just as the eukaryotic acetyltranferase and transcription factors. However, in this case, the auto-acetylation resulted in the inactivation of Mt-Acs

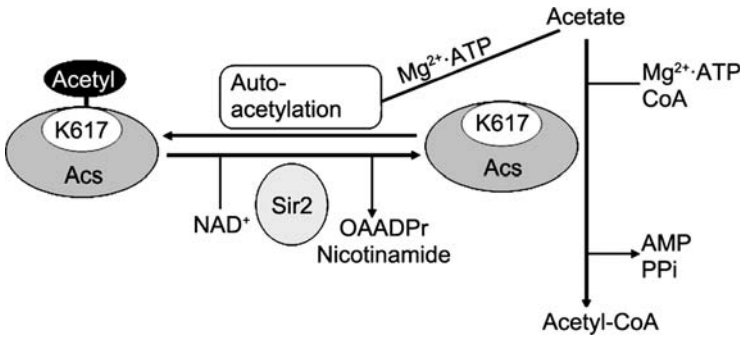

Figure 8 Schematic illustration of Mt-Acs regulation by reversible lysine acetylation of Lys-617 Acs undergoes auto-acetylation with acetate as the acetyl donor, which results in the decrease of its acetyl-CoA synthetase activity. The NAD^+-dependent deacetylase Sir2 activated Acs by removing the acetyl group, releasing 2′-*O*-acetyl-ADP-ribose (OAADPr) and nicotinamide. Unacetylated Acs is competent for the conversion of acetate to acetyl-CoA.

activity, as measurement of the specific activity of Mt-Acs with different levels of acetylation indicated.

Very interestingly, we found that CoA, the substrate of Mt-Acs for AcCoA formation, effectively inhibited auto-acetylation. One possible explanation is that CoA may compete with Mt-Acs protein for acetyl group. When exposed to acetate and ATP, Mt-Acs synthesized AcAMP [14]. If CoA is not available or the concentration of CoA is low *in vivo*, Mt-Acs can transfer acetyl group from AcAMP to itself, resulting in auto-acetylation and inactivation. When CoA is available, the acetyl group is donated to CoA forming AcCoA.

In *S. enterica*, acetylation of Acs by the Pat inactivated the enzyme, whereas deacetylation by the sirtuin CobB reactivated it [11,13]. Protein BLAST search of *M. tuberculosis* Pat homolog was performed, but no such homolog could be found. Further studies are necessary to identify the Mt-Acs acetyltransferase. On the other hand, our data showed that Mt-Sir2, an NAD^+-dependent deacetylase of *M. tuberculosis*, contributed to the reversibility of Mt-Acs auto-acetylation, and resulted in activation of the enzymatic activity. A schematic overview of the regulation of Mt-Acs by reversible lysine acetylation was given in **Fig. 8**.

In conclusion, we have demonstrated a method for producing active recombinant form of *M. tuberculosis* Acs. The availability of Mt-Acs protein should thus facilitate the 3D structure studies and further *in vivo* experiments for investigating its physiological roles in *M. tuberculosis* central metabolism.

Supplementary Data

Supplementary data are available at *ABBS* online.

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