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Acetylation of acetyl-CoA synthetase from *Mycobacterium tuberculosis* leads to specific inactivation of the adenylation reaction ☆

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ABSTRACT

Acetyl-CoA synthetase (ACS) catalyzes the formation of AcCoA from acetate, ATP and Coenzyme A, allowing the organism to grow on acetate as the sole carbon source. ACS was the first enzyme in *Mycobacterium tuberculosis* shown to be regulated by posttranslational acetylation by the cAMP-dependent protein acetyltransferase. This modification results in the inactivation of the enzyme and can be reversed in the presence of NAD^+ and a mycobacterial sirtuin-like deacetylase. In this study we characterize the kinetic mechanism of *Mt*ACS, where the overall reaction can be divided into two half-reactions: the acetyl-adenylate forming reaction and the thiol-ligation reaction. We also provide evidence for the existence of the acetyl-adenylate intermediate via ^{31}P NMR spectroscopy. Furthermore, we dissect the regulatory role of K617 acetylation and show that acetylation inhibits only the first, adenylation half-reaction while leaving the second half reaction unchanged. Finally, we demonstrate that the chemical mechanism of the enzyme relies on a conformational change which is controlled by the protonation state of aspartate 525. Together with our earlier results, this suggests a degree of regulation of enzyme activity that is appropriate for the role of the enzyme in central carbon metabolism.

Introduction

Acetyl-CoA (AcCoA) is an important intermediate in many pathways of intermediary metabolism, including fatty acid biosynthesis and oxidation, the TCA-cycle and the glyoxylate shunt [1]. AcCoA can be synthesized from pyruvate by pyruvate dehydrogenase via the glycolytic sequence [2], or *de novo* by acetyl-CoA synthetase (ACS) from acetate (Ac) and Coenzyme A (CoA) in an ATP-dependent manner (Scheme 1) [3]. The latter enables the bacterium to survive on a medium that contains acetate as a sole carbon source. ACS is a member of the adenylate-forming enzyme superfamily, among which are aminoacyl-tRNA synthetases, the nonribosomal peptide and polyketide synthetases and the fatty acyl CoA- and ACP-ligases. Members of this family are structurally characterized by a small C-terminal domain and a larger N-terminal domain that form a cleft that includes the active site of the enzyme [3]. *Mt*ACS[1] was recently shown to be the first enzyme in *Mycobacterium tuberculosis* to be regulated by reversible posttranslational acetylation [4]. Mycobacteria contain a single cAMP-dependent protein acetyltransferase that catalyzes the acetylation of K617 of *Mt*ACS, resulting in inactivation of the enzyme. This acetylation is reversed in the presence of NAD^+ and a mycobacterial sirtuin-like deacetylase. This regulation suggests an important role for ACS in the modulation of the energy pool in mycobacteria.

In *Escherichia coli*, ACS is essential for growth in high-density cell culture. Under these conditions, glucose becomes limiting, and continued growth requires ACS to convert acetate into AcCoA which can be converted into hexoses via the glyoxylate shunt and gluconeogenesis. Acetate is a weakly lipophilic acid that can easily penetrate the membrane, acidify the cytoplasm, increase the osmotic pressure within the cell, and interfere with methionine biosynthesis [5]. One of roles of ACS is thus to convert acetate into AcCoA thereby reducing the toxicity caused by acetate in the medium [5]. It is possible that *M. tuberculosis* has to deal with similar environmental stresses when it resides within the macrophage phagosome, granuloma or tubercule, which are unique habitats for the organism [6]. A recently published paper described an increase in the levels of different metabolites within the granuloma, one of which was acetate. This finding suggests a change in the selection of different metabolic pathways by *M. tuberculosis* at different stages of the disease [7]. *M. tuberculosis* thus has to adapt to this changing external environment, probably in a similar manner to *E. coli* by upregulating ACS activity. However, in contrast

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[1] *Abbreviations used:* *Mt*ACS, *Mycobacterium tuberculosis* Acetyl-CoA synthetase; AcACS, acetylated-acetyl CoA synthetase; Ac, acetate; CoA, Coenzyme A; AcCoA, acetyl Coenzyme A; AcAMP, acetyl-adenylate.

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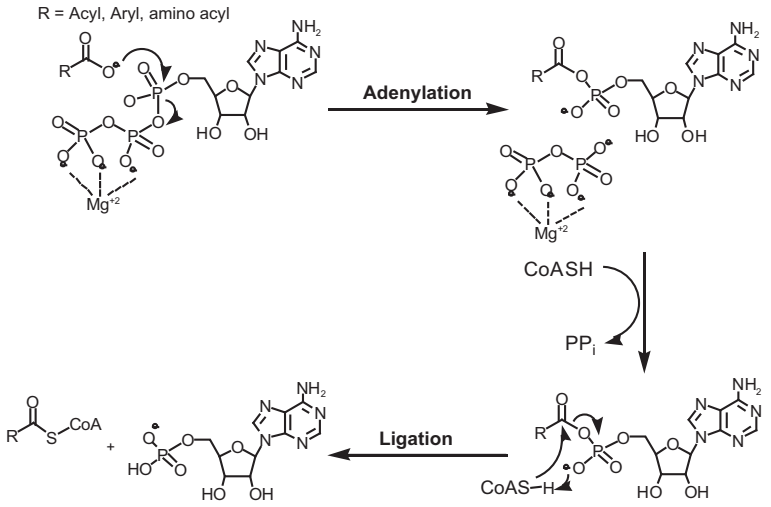

Scheme 1.

to *E. coli*, *M. tuberculosis* can use glucose and acetate simultaneously as a carbon source and does not exhibit diauxonic growth [8]. Thus it is very likely that ACS can both reduce the toxicity of Ac and serve as a gateway for Ac into central carbon metabolism.

Despite its obvious importance in allowing for growth on acetate alone, or the observations that ACS is posttranslationally acetylated in a number of microorganisms [9], including *M. tuberculosis*, there are no detailed mechanistic studies of the enzyme. As a member of the adenylate-forming acyl-CoA synthetases, the kinetic mechanism is predicted to be a bi-uni-uni-bi ping pong mechanism, where the overall reaction can be divided into two half reactions (Scheme 1): acetyl-adenylate formation and the thiol ligation reaction leading to AcCoA and AMP formation. In this study we provide evidence for the kinetic mechanism and the existence of the acetyl-adenylate intermediate via ^{31}P NMR spectroscopy. We show that acetylation prevents only the first adenylation half reaction while leaving the formation of AcCoA from the acetyladenylate and CoA unchanged. We also characterize the chemical mechanism of *M. tuberculosis* ACS by using pH rate profiles and show that a conformational change between the adenylate forming and thioester forming conformations is dependent on the ionization state of D525.

Materials and methods

Materials

All chemicals were purchased from Sigma–Aldrich chemical company.

Expression and purification of MtACS

The plasmid pET28a:*Mt*ACS was transformed into T7 express *E. coli* cells. A single colony was selected, grown in 25 ml of LB medium supplemented with 30 μg/ml kanamycin, and these cells were used to inoculate 6L the same media. The cells were grown to mid-log phase at 37 °C, and induced by the addition of 1 mM IPTG for 4.5 h at 25 °C. The cells were harvested by centrifugation and the pellet was resuspended in buffer containing 50 mM sodium phosphate buffer, pH 8.0 containing 300 mM NaCl (buffer A). The cells were lysed in an EmulsiFlex-C3 homogenizer (Avestin) and centrifuged for 1 h at 38,000*g*. The supernatant was loaded onto Ni–NTA column (QIAGEN), that was pre-equilibrated with buffer A. The unbound proteins were eluted with 5 column volumes of 10 mM imidazole in buffer A, and eluted with a 20 column-volume linear imidazole gradient, from 10 to 250 mM imidazole. Fractions containing pure *Mt*ACS were determined using SDS–PAGE followed by Coomassie Blue staining. These fractions were pooled and dialyzed against 100 mM HEPES, pH 7.5 containing 100 mM KCl, followed by concentration with an Amicon concentrator with a 30Kd cutoff.

Site-directed mutagenesis

The D525N mutation was introduced into pET28a+/ACS by using pfu ultra polymerase (from Stratagene) and the following primers: FW-GGTACTAGGCCGCATCAACGACGTGATGAACGTGTCCGGG, RV-CCCGGACACGTTCATCACGTCGTTGATGCGGCCTAGTACC, where the underlined residues are the site of the change.

Enzyme activity assay

Initial reaction velocities of *Mt*ACS were assayed spectrophotometrically by coupling the formation of AMP to the reactions of myokinase, pyruvate kinase and lactate dehydrogenate, and following the decrease in absorbance of NADH at 340 nM (ε_{340} - = 6220 M^{-1} s^{-1}). All reactions were performed at 25 °C using a Shimadzo UV-2450 spectrophotometer. A typical reaction mixture contained 100 mM HEPES, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM PEP, 0.1 mM NADH, 6 units of myokinase, 6 units of pyruvate kinase, 6 units of lactate dehydrogenase, *Mt*ACS at a final concentration of 100 nM and variable concentrations of the substrates: Ac, ATP and CoA. The reaction mixtures were incubated for 1 min at room temperature and reactions were initiated by the addition of Coenzyme A. Reactions were followed for 3–5 min and velocities were calculated assuming 2 molecules of NADH were oxidized for each AcCoA molecule formed.

Initial velocity experiments

Kinetic constants for ATP, Ac and CoA were determined by using at least five different concentrations of each substrate while saturating with the others (~1 mM). The kinetic parameters for each substrate determined by fitting each substrate saturation curve to Eq. (1) with Sigma Plot 11.0:

$$v = (VA)/(K + A) \quad (1)$$

where V is maximal velocity, A is substrate concentration, and K is the Michaelis–Menten constant for the variable substrate, A. Initial velocity patterns were determined at various concentrations of one