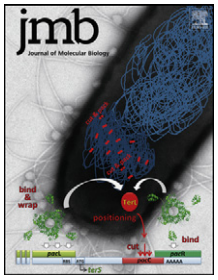

Studying the Lysine Acetylation of Malate Dehydrogenase

Sumana Venkat[1,2], **Caroline Gregory**[3], **Jourdan Sturges**[3], **Qinglei Gan**[1] and **Chenguang Fan**[1,2]

*1 - **Department of Chemistry and Biochemistry,** University of Arkansas, Fayetteville, AR 72701, USA*
*2 - **Cell and Molecular Biology Program,** University of Arkansas, Fayetteville, AR 72701, USA*
*3 - **Department of Biological Sciences,** University of Arkansas, Fayetteville, AR 72701, USA*

***Correspondence to Chenguang Fan:** Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA. cf021@uark.edu*
http://dx.doi.org/10.1016/j.jmb.2017.03.027
Edited by Achillefs Kapanidis

Abstract

Protein acetylation plays important roles in many biological processes. Malate dehydrogenase (MDH), a key enzyme in the tricarboxylic acid cycle, has been identified to be acetylated in bacteria by proteomic studies, but no further characterization has been reported. One challenge for studying protein acetylation is to get purely acetylated proteins at specific positions. Here, we applied the genetic code expansion strategy to site-specifically incorporate N^{ε}-acetyllysine into MDH. The acetylation of lysine residues in MDH could enhance its enzyme activity. The *Escherichia coli* deacetylase CobB could deacetylate acetylated MDH, while the *E. coli* acetyltransferase YfiQ cannot acetylate MDH efficiently. Our results also demonstrated that acetyl-CoA or acetyl-phosphate could acetylate MDH chemically *in vitro*. Furthermore, the acetylation level of MDH was shown to be affected by carbon sources in the growth medium.

Introduction

The acetylation of lysine residues is one of the most common post-translational modifications of proteins that regulate diverse cellular functions including DNA–protein interaction, transcription activity, protein stability, stress response, apoptosis, cellular differentiation, and energy metabolism [1–13]. The abnormality of acetylation modifications is associated with diabetes, cardiovascular diseases, cancers, and neurodegenerative disorders [14–24]. During the last decade, proteomic studies have identified lysine acetylation in thousands of eukaryotic proteins, which significantly expanded our knowledge of protein acetylation [25–27].

Interestingly, protein acetylation in bacteria starts to attract attention in the last 5 years [28–35]. A series of proteomic studies have identified hundreds of bacterial proteins with lysine acetylation, including metabolic enzymes, stress response proteins, regulator of chemotaxis, chaperones, and transcription and translation factors [36–47]. However, few studies have been performed to further characterize these acetylated proteins; take *Escherichia coli* as an example, only six proteins with lysine acetylation were studied, including acetyl-CoA synthetase [48–51]; CheY, the regulator of bacterial chemotaxis [52–54]; RcsB, the regulator of capsule synthesis [55–57]; RNase R [58]; *N*-hydroxyarylamine *O*-acetyltransferase [59]; and α-subunit of RNA polymerase [60,61]. One challenge for studying lysine acetylation is that it is difficult to synthesize purely acetylated proteins at specific sites by most classic methods. To solve this problem, researchers applied the genetic code expansion strategy. Rather than adding acetyl group after protein translation, this approach uses an orthogonal pair of an engineered pyrrolysyl-tRNA synthetase variant and its cognate tRNA from *Methanosarcinaceae* species to co-translationally direct the incorporation of N^{ε}-acetyllysine (AcK) in response to a stop codon at desired positions in target proteins [62–65].

Malate dehydrogenase (MDH), a widely distributed enzyme catalyzing the conversion of oxaloacetate and malate, plays key roles in many important

metabolic pathways including the tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis, and the exchange of metabolites between cytoplasm and subcellular organelles [66,67]. Previous studies have shown that the acetylation of lysine residues in mammalian MDHs is involved in the cross-talk mechanisms between adipogenesis and the intracellular energy level [68–70]. However, the acetylation of bacterial MDH has not been characterized before. Here, we applied the genetic code expansion strategy to study the lysine acetylation of MDH in *E. coli*. We also used the same strategy to study human MDH as comparison.

Results

Selecting acetylation sites in MDHs

Recently, several proteomic studies have identified lysine acetylation in *E. coli* MDH (eMDH), and lysine residues K99 and K140 were identified to be acetylated in all of these reports [37–39,46,47]. So, we chose these two positions to incorporate AcK. In human cells, there are two MDH isozymes, the cytosol MDH1 and mitochondrial MDH2 [71]. Human MDH2 (hMDH2) has higher homology to eMDH than hMDH1 does [72]. Previous proteomic studies showed that hMDH2 has four lysine residues acetylated at positions K185, K301, K307, and K314 [73]. So, we also chose these four positions to incorporate AcK as comparison. Interestingly, the alignment of eMDH and hMDH2 showed different patterns: the acetylation sites in eMDH are at the middle part of the primary sequence, while those in hMDH2 are mainly at the C terminus (Fig. S1). The acetylation site K185 in hMDH2 has its counterpart in eMDH at position K162, which was also selected to incorporate AcK as a control.

Site-specifically incorporating lysine acetylation

We incorporated AcK at selected positions mentioned above in both eMDH and hMDH2 by our recently optimized AcK incorporation system, which has an optimized $tRNA^{Pyl}$ with better binding with *E. coli* elongation factor (EF-Tu), thus increasing the incorporation efficiency [65]. The AcK was genetically encoded by an amber stop codon (TAG), which was introduced by site-directed mutagenesis. Previous studies showed that the K12-derived strains have substantially higher acetylation than B-strain-derived BL21 cells during growth [39]. To lower the background of non-specific acetylation at other lysine residues, we used BL21 (DE3) strain as the expression host. The incorporation of AcK was confirmed by both western blotting (Fig. 1a) and mass spectrometry (MS; Fig. S2–S8).

The lysine acetylation of MDH increases its enzyme activities

The enzyme activities of eMDH and hMDH2 and their acetylated variants were measured (Fig. 1b). For eMDH, the acetylation at positions K99 and K140 increased the enzyme activity by 2.3- and 3.4-fold, individually, which is consistent with our previous study [65], while the acetylation at the position K162 had little effect. Our previous study also showed that doubly acetylated eMDH at both positions K99 and K140 had sixfold higher enzyme activity than that of wild-type eMDH [65]. For hMDH2, only the acetylation at the position K307 increased the enzyme activity by 3.9-fold, while others had no obvious effects.

Steady-state kinetic analyses were performed with wild-type MDHs and their acetylated variants (Table 1). The lysine acetylation appeared to not affect the K_M values of both substrates, NAD^+ and malate, indicating that the increase of enzyme activities results mainly from the improvement of the overall turnover.

CobB can deacetylate acetylated MDHs at specific positions

Lysine deacetylases remove the *N*-acetyl amide moieties and can be broadly divided into two families according to their reaction mechanism: hydrolytic deacetylases and NAD^+-dependent deacetylases, which are also named as sirtuins [32,74]. The CobB protein, a sirtuin family member, was found in many bacteria including *E. coli* and *Salmonella* [48,51]. Recently, YcgC has been confirmed to be a hydrolytic deacetylase and targets a distinct set of substrates from *E. coli* CobB, representing a novel family of prokaryotic deacetylases [75].

To determine the deacetylation activity of the CobB protein on acetylated MDHs, researchers used K12-derived *E. coli* TOP10 strain with higher acetylation levels for *in vivo* tests [39]. The genes of eMDH and hMDH2 with C-terminal His_6 tags were expressed and purified in wild-type or Δ*cobB* cells, individually. Western blotting was used for detecting the acetylation (Fig. 2a). The deletion of *cobB* gene increased the acetylation levels of both eMDH and hMDH2, indicating that CobB could deacetylate their lysine acetylation *in vivo*. We also measured the enzyme activities of MDHs from wild-type or Δ*cobB* cells (Fig. 2b). The MDHs purified from Δ*cobB* cells had higher enzyme activities, which was consistent with the western blotting results, as lysine acetylation could increase MDH activities.

We also performed *in vitro* deacetylation experiments. The optimized AcK incorporation system mentioned above was used to generate site-specially acetylated eMDH at positions K99, K140, and K162, individually, and acetylated hMDH2s at positions K185, K301, K307, and K314, respectively. All these