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Characterizing Lysine Acetylation of Isocitrate Dehydrogenase in *Escherichia coli*

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Abstract

The *Escherichia coli* isocitrate dehydrogenase (ICDH) is one of the tricarboxylic acid cycle enzymes, playing key roles in energy production and carbon flux regulation. *E. coli* ICDH was the first bacterial enzyme shown to be regulated by reversible phosphorylation. However, the effect of lysine acetylation on *E. coli* ICDH, which has no sequence similarity with its counterparts in eukaryotes, is still unclear. Based on previous studies of *E. coli* acetylome and ICDH crystal structures, eight lysine residues were selected for mutational and kinetic analyses. They were replaced with acetyllysine by the genetic code expansion strategy or substituted with glutamine as a classic approach. Although acetylation decreased the overall ICDH activity, its effects were different site by site. Deacetylation tests demonstrated that the CobB deacetylase could deacetylate ICDH both *in vivo* and *in vitro*, but CobB was only specific for lysine residues at the protein surface. On the other hand, ICDH could be acetylated by acetyl-phosphate chemically *in vitro*. And *in vivo* acetylation tests indicated that the acetylation level of ICDH was correlated with the amounts of intracellular acetyl-phosphate. This study nicely complements previous proteomic studies to provide direct biochemical evidence for ICDH acetylation.

Graphical Abstract

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Keywords

post-translational modification; carbon flux; tricarboxylic acid cycle; genetic code expansion; glyoxylate bypass

INTRODUCTION

The family of isocitrate dehydrogenases (ICDHs) catalyzes the reaction of converting isocitrate to α -ketoglutarate and carbon dioxide in two steps: the oxidation of isocitrate to the intermediate oxalosuccinate and the decarboxylation of its β -carboxyl group to α -ketoglutarate [1]. Depending on the electron acceptor, ICDHs have two distinct classes: NAD⁺-dependent ICDHs (EC 1.1.1.41) and NADP⁺-dependent ICDHs (EC 1.1.1.42) [2, 3]. In human cells, NAD⁺-dependent ICDH3 is located at the mitochondrial matrix and plays the role in energy production through the tricarboxylic acid (TCA) cycle, while NADP⁺-dependent ICDH1 and ICDH2 are found in the cytosol and the mitochondria respectively, shuttling electrons between the cytosol and the mitochondria [4]. Due to the important role in generating NADPH for cellular defense against oxidative damage, NADP⁺-dependent ICDHs have been identified to be associated with a number of serious human diseases such as cancer and leukemia [5, 6]. Different from eukaryotic cells, *Escherichia coli* only has the NADP⁺-dependent form of ICDH [7, 8], which plays both roles in generating energy through the TCA cycle and producing NADPH to protect cells from oxidative damage.

E. coli ICDH has been known to be regulated by phosphorylation for more than 30 years [9, 10]. The phosphorylated serine residue at position 113 completely inactivates the enzyme, while dephosphorylation restores the impaired activity [11]. Such reversible phosphorylation is catalyzed by a single enzyme, the bifunctional ICDH kinase/phosphatase, which is essential for *E. coli* growth on acetate as sole carbon source [12]. Since isocitrate is the common substrate of ICDH in the TCA cycle for energy production and the isocitrate lyase (ICL) in the glyoxylate shunt for biosynthetic intermediate generation, regulation of ICDH is critical in controlling the ratio of carbon destined for cell growth and energy production [13–15].

As another widely-distributed post-translational modification in nature, reversible acetylation of lysine residues is essential for a wide range of biological processes such as gene expression, energy metabolism, and cell signaling. Although first identified and well studied in eukaryotic cells, more and more evidence has indicated that lysine acetylation is also involved in a number of prokaryotic cellular functions [16–30]. Interestingly, proteomic studies have demonstrated that metabolic enzymes including ICDHs are favorable targets for lysine acetylation in both eukaryotes and prokaryotes [31]. Previous studies of eukaryotic NADP⁺-dependent mitochondrial ICDH2 showed that it is regulated by lysine acetylation for defending reactive oxygen species under calorie restriction [32, 33]. However, *E. coli* ICDH has no sequence identity with eukaryotic ICDH2, so the effect of lysine acetylation on *E. coli* ICDH still remains unclear. Recently, we have characterized lysine acetylation of another TCA cycle enzyme, malate dehydrogenase (MDH) in *E. coli* [34]. Our results showed that lysine acetylation could increase the MDH activity and was regulated by the

CobB deacetylase and intracellular acetyl-phosphate, as well as the carbon source in growth media. In this study, our results demonstrated that lysine acetylation of ICDH has distinct effects but also shares common features with the acetylation of MDH. To our best knowledge, this is the first biochemical characterization of lysine acetylation of bacterial-type NADP⁺-dependent ICDH.

RESULTS

Site-specifically incorporating lysine acetylation into ICDH

Proteomic studies of *E. coli* have identified totally more than 20 lysine residues to be acetylated in ICDH [35–44]. However, the sets of acetylation sites from these studies are distinct from each other, mostly because of the differences in *E. coli* strains, growth conditions, acetylated peptide detection methods, and mass spectrometry resolutions. Thus, we chose lysine residues for later studies based on both proteomic [35–44] and structural studies [45–52] by three criteria: 1) those that have been identified in more than four independent proteomic studies; 2) those that are located at the active site or subunit interfaces; 3) those that are conserved among different organisms. Based on these standards, eight lysine residues (K55, K100, K142, K177, K230, K235, K242, and K350) were selected. Among them, only K100 has not been identified to be acetylated before, but it was reported to be succinylated instead [44, 53]. We chose it as a control because of its essential role in catalysis and substrate binding [52].

Then we applied the genetic code expansion approach to incorporate acetyllysine directly into those selected positions by our recently optimized protocol individually [54]. This approach utilizes an engineered *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase [55] and an optimized cognate tRNA^{pyl} [56] to read through the TAG stop codon in the gene of ICDH introduced by site-directed mutagenesis as acetyllysine, thus producing homogeneously acetylated ICDH variants at selected sites [57, 58]. To reduce the background of non-specific acetylation at other lysine residues, we used the BL21 (DE3) strain as the expression host which was shown to have significantly lower levels of protein acetylation than *E. coli* K12-derived strains [37]. Indeed, the wild-type ICDH purified from BL21 (DE3) cells had no detectable acetylation by western blotting, while site-specifically acetylated ICDH variants all had clear bands against the acetyllysine antibody (Figure 1a). Mass spectrometry (MS) analyses confirmed that the incorporation of acetyllysine was at selected positions respectively (Figure S1–S9).

Determining the effect of lysine acetylation on the ICDH activity

The enzyme activities of ICDH and its acetylated variants were measured (Figure 1b). The effect of acetylation on the enzyme activity was different site by site. Acetylation at K55 or K350 significantly increased the activity, while acetylation at K142, K177, or K242 only had small enhancement. On the other hand, acetylation at K235 lost about 40% of the activity, while acetylation at K100 or K230 almost completely eliminated the enzyme activity.

As a classic approach, glutamine is commonly used as a mimic of acetylated lysine. Thus, we also substituted selected lysine residues with glutamine respectively, and measured the

activities of those K-to-Q variants as a comparison with the direct acetyllysine incorporation approach. Interestingly, the results from glutamine substitution were quite different from those by direct acetyllysine incorporation (Figure 1b). Substituting lysine with glutamine decreased enzyme activities at all tested positions. Glutamine substitution at K100 or K230 abolished the enzyme completely which was similar to the acetyllysine incorporation approach, but results for other positions were quite different from two approaches. For example, replacement of K55 by glutamine decreased the enzyme activity dramatically, but direct incorporation of acetyllysine at K55 significantly increased the activity (Figure 1b).

To illustrate how acetylation affect the enzyme activity, steady-state kinetic analyses were performed for the wild-type ICDH and its acetylated variants (Table 1). Acetylation at K55 or K350 increased the turnover number, while acetylation at K142, K177, or K242 had no significant effects on kinetic parameters. On the other hand, acetylation at K100 decreased the catalytic efficiency by 10⁶-fold, consistent with the previous study in which K100 was replaced with Met, Gln, or His [52]. Acetylation at K230 dramatically decreased both the turnover number and the affinity of isocitrate which aligned with previous mutagenesis studies on ICDH [49, 59], while acetylation of K235 halved the turnover number without affecting binding of both substrates.

Proteomic studies indicated that *E. coli* ICDH could be multiply acetylated simultaneously [35–44], so possible cooperative effects of multiple lysine acetylation on ICDH were tested. Starting with simpler cases, we applied the genetic code expansion strategy to incorporate acetyllysine (AcK) at two sites of selected lysine residues simultaneously. As a representative, K55, of which acetylation increased the enzyme activity significantly, was chosen to be acetylated in all variants. Totally seven dual-acetylated ICDH variants were generated (ICDH-55AcK/100AcK, ICDH-55AcK/142AcK, ICDH-55AcK/177AcK, ICDH-55AcK/230AcK, ICDH-55AcK/235AcK, ICDH-55AcK/242AcK, and ICDH-55AcK/350AcK). Both western blotting and mass spectrometry analyses confirmed the simultaneous incorporation of acetyllysine at selected positions (Figure 2a, Figure S10–S17). Then we measured the enzyme activities of these dual-acetylated variants (Figure 2b). However, the improving effect of acetylation at K55 could not recover impaired activities caused by acetylation at K100 and K230, implying their essential roles in catalysis or substrate binding.

Due to the existence of endogenous deacetylases in cells, lysine residues usually are not acetylated completely. Thus, besides above *in vitro* experiments in which lysine residues were homogeneously acetylated, we also performed *in vivo* studies which could reflect the real situation in living cells. The *E. coli* K12-derived BW25113 strain was used for all *in vivo* experiments in this study (unless indicated). Firstly, we compared the acetylation levels of ICDH purified from BW25113 cells grown in LB media or M9 media with 0.2% glucose. Cells were grown at 37°C for 8 hours after inoculation. The native ICDH was purified by the established protocol with 50 mM nicotinamine (the deacetylase inhibitor) in all purification buffers [60]. The acetylation of purified ICDHs was determined by western blotting (Figure 3a). ICDH purified from LB media had no detectable acetylation, while that from glucose media had a clear band, indicating glucose could induce the acetylation of ICDH *in vivo*, which is consistent with previous proteomic studies [37, 41, 43]. Then we measured their

enzyme activities (Figure 3b). ICDH purified from glucose media with a higher acetylation level lost about 60% of the activity compared to that from LB media. We were not surprised at these results, because our *in vitro* experiments above showed that the acetylation at key residues such as K100 and K230 almost impaired the enzyme activity completely which could not be recovered by acetylation at other positions even those like K55 of which acetylation could increase the enzyme activity (Figure 2b).

Deacetylation of ICDH

The acetyl on the ϵ -amino group of lysine residues is stable, and its removal needs a category of enzymes called lysine deacetylases [61, 62]. Till now, there are only two known lysine deacetylases identified in *E. coli*: the CobB protein, a NAD⁺-dependent sirtuin class deacetylase [63–65], and the YcgC protein, a NAD⁺-independent serine hydrolase family member with a distinct set of substrates from CobB [66]. To determine the deacetylation of ICDH by CobB and YcgC, the acetylation levels of native ICDHs purified from wild-type, *ycgC*, and *cobB* of BW25113 cells grown in M9 media with 0.2% glucose were compared by western blotting (Figure 4a). The deletion of *cobB* increased the acetylation level of ICDH, consistent with previous quantitative proteomic studies [39–42], while inactivation of *ycgC* had no obvious effect.

A previous study showed that inactivation of the *cobB* gene increased the acetyl-phosphate concentration in cells [39], and acetyl-phosphate could increase protein acetylation globally [37, 41]. Thus, although the deletion of the *cobB* gene increased the acetylation level of ICDH (Figure 4a), we cannot conclude that CobB can deacetylate ICDH directly. To provide direct evidence, we performed *in vitro* deacetylation experiments. The site-specifically acetylated ICDH variants generated by the genetic code expansion strategy were treated with the purified CobB protein, respectively. Western blotting was used for detecting the acetylation (Figure 4b). The results showed that CobB was only specific for position 55, 142, 177, or 350. After 2-hour treatment of CobB, no detectable acetylation was observed for these positions by western blotting, while acetylation at other positions were not changed obviously, which could explain the detectable acetylation with an intact *cobB* gene in wild-type BW25113 cells (Figure 3a). Those selected lysine residues were mapped onto the crystal structure of ICDH [46] (Figure S18). CobB-sensitive sites (K44, K142, K177, and K350) are all located at ICDH surfaces, while CobB-resistant sites (K100, K230, K235, and K242) are internal and close to the active site. Although the CobB substrate specificity is varied by different criterions, a common feature is to be located at protein surfaces [37, 42]. The same *in vitro* deacetylation experiments were performed for the YcgC protein (Figure 4c). Consistent with *in vivo* experiments, no significant changes were observed after 2-hour treatment of YcgC at all sites tested. RutR, the known substrate of YcgC [66], was used as a positive control to show that YcgC was active in this study (Figure S19). We also performed enzyme assays to measure the activities of those site-specifically acetylated ICDH variants after CobB and YcgC treatment (Figure 4d). Treatment of the variant acetylated at position 55, 142, 177, or 350 with the CobB protein decreased its activity to the level of wild-type enzyme, consistent with above western blotting results which showed that CobB was specific for those sites.

The acetylation of ICDH

Currently, it is believed that lysine acetylation in *E. coli* has two major mechanisms: the acetyl-CoA-dependent enzymatic process and the acetyl-phosphate-dependent chemical reaction [16–22]. YfiQ is the only known protein acetyltransferase in *E. coli* which belongs to the Gcn5-related N-acetyltransferase family [67, 68]. To determine the acetylation of ICDH by YfiQ, the acetylation levels of native ICDHs purified from wild-type, *yfiQ*, *cobB*, and *yfiQ cobB* of BW25113 cells grown in M9 media with 0.2% glucose were compared by western blotting (Figure 5a). The deletion of *yfiQ* did not decrease the acetylation level of ICDH expressed in cells with or without the *cobB* background, indicating that ICDH is not the substrate of YfiQ *in vivo*, which is consistent with data from proteomic studies [37, 40–42]. To provide direct evidence, we also performed *in vitro* acetylation experiments. The wild-type ICDH expressed and purified from BL21 (DE3) cells was treated with purified YfiQ and 0.2 mM acetyl-CoA. After 2-hour incubation, ICDH had no detectable acetylation, indicating that ICDH is not the substrate of YfiQ *in vitro* either (Figure S20).

Then, we tested the chemical acetylation by acetyl-phosphate alone *in vitro*. The wild-type ICDH expressed and purified from BL21 (DE3) cells was treated with acetyl-phosphate at concentrations of 200 μ M, 3 mM, and 12 mM, corresponding to estimated intracellular acetyl-phosphate concentrations at the exponential phase, the stationary phase, and the *ackA* background which accumulates acetyl-phosphate, respectively [37, 69, 70]. Western blotting was used to determine acetylation levels (Figure 5b). Clearly, acetyl-phosphate could chemically acetylate ICDH in both dose- and time-dependent manners *in vitro* which aligned with proteomic studies [37, 41, 43]. The acetyl-phosphate-treated ICDH was then analyzed by LC-MS/MS to determine acetylated lysine sites. Besides the eight selected sites in above *in vitro* studies, we identified additionally acetylated lysine residues at positions 4, 20, 47, 73, 166, 174, 265, 273, 278, 378, and 387 (Figure S21–S31). Most of them are located at the protein surface which is consistent with the specificity of acetyl-phosphate-dependent acetylation [41, 43]. But we did not detect acetylation at positions 12, 58, 186, 199, 267, and 401 which have been reported by proteomic studies before [31]. Although this could result from differences in strains, growth conditions, and mass spectrometry resolutions, it also indicates that acetylation at those sites may need additional cofactors, coenzymes, or unknown protein acetyltransferases.

Reversely, among those acetylated lysine residues detected in *in vitro* acetylation experiments, K47, K73, and K278 as well as K100 have never been identified to be acetylated in any proteomic studies before, indicating the differences between the *in vitro* study and the *in vivo* process. Thus, we performed *in vivo* tests for acetyl-phosphate-dependent acetylation of ICDH. Acetyl-phosphate is the intermediate of the phosphotransacetylase (Pta) – acetate kinase (AckA) pathway [71]. Pta catalyzes the reversible conversion between acetyl-CoA and acetyl-phosphate, while AckA catalyzes the reversible conversion between acetate and acetyl-phosphate. When cells are grown in media without using acetate as carbon source, inactivation of *pta* will block the acetyl-phosphate production, while *ackA* accumulates acetyl-phosphate [71]. The acetylation levels of native ICDHs purified from wild-type, *pta*, and *ackA* of BW25113 cells grown in M9 media

with 0.2% glucose were compared by western blotting (Figure 5c). As expected, acetylation of ICDH was correlated with intracellular concentrations of acetyl-phosphate. *ackA* increased the acetylation level of ICDH which is consistent with proteomic studies [37, 41]. Although our *in vitro* studies demonstrated that acetyl-phosphate could acetylate ICDH without any enzymes, we could not exclude the existence of specific protein acetyltransferases for ICDH acetylation *in vivo* because of the different sets of acetylated lysine residues detected by *in vitro* and *in vivo* approaches.

DISCUSSION

By site-specifically incorporating acetyllysine into selected lysine positions, we provided direct biochemical evidence for the effect of acetylation on ICDH site by site. Besides the key residues K100 and K230 in catalysis and substrate binding which have already been characterized by mutational, kinetic, and structural studies before [46, 49, 50, 52, 59], we also showed that acetylation of K55 and K350 could increase the activity (Figure 1a) which is totally unknown before. Although crystal structures of the ICDH K55AcK and ICDH K350AcK variants are needed to demonstrate the mechanism in detail, we could still use existing ICDH crystal structures to propose acetylation effects (Figure 6). K350 is located at the entrance of the active site, and is close to the substrate NADP⁺. Although K350 does not contact with NADP⁺ directly, the removal of the positive charge of the lysine side chain may facilitate NADP⁺ binding as shown in the kinetic analysis (Table 1). On the other hand, K55 is located at the back side of the active site, but it interacts with three hydrophobic residues (L375, I413, and M416) in two helices which form part of the active site. Acetylation neutralizes the positive charge of K55 side chain, thus facilitating its interactions with those hydrophobic side chains which may induce conformational changes to improve the catalysis.

During growth of *E. coli* on acetate, the glyoxylate shunt pathway bypasses two steps in the TCA cycle, generating succinate, malate, and free coenzyme A from isocitrate to replenish central metabolism with biosynthetic precursors [72]. The first enzyme in the glyoxylate bypass, isocitrate lyase (ICL), is the direct competitor of ICDH for isocitrate. The partition of carbon flux between ICDH and ICL at the junction of isocitrate is well controlled to balance energy production and cell growth in different growth stages and conditions [14, 15, 73]. As the first prokaryotic enzyme known to be regulated by phosphorylation, ICDH has been well studied on its regulation by the bifunctional ICDH kinase/phosphatase [9, 12, 74]. ICL was also shown to be phosphorylated at a histidine residue [75–77], but it is more sensitive to the concentration of isocitrate in cells [14, 15, 73]. Interestingly, Both ICDH and ICL are acetylated at multiple lysine residues which could be induced by glucose in growth media [37, 40, 41, 43]. A previous study showed that ICL activity decreased with acetylation [40]. And our study here indicated that acetylation could also impair the ICDH activity (Figure 3b). However, the net effect of lysine acetylation on dividing isocitrate into two pathways needs to be determined in future studies.

Another open question is whether phosphorylation and acetylation have crosstalk for regulating ICDH and IDL for the carbon flux partition. Firstly, proteomic data showed that the bifunctional ICDH kinase/phosphatase is also acetylated at multiple lysine residues [31], but these acetylation targets have not been confirmed and further characterized. Secondly,

the acetylation of ICDH may affect its own phosphorylation, since many acetylated lysine residues studied in this work are located close to S113 which is the key residue for reversible phosphorylation [51, 78]. And our recently developed genetic incorporation system for simultaneous acetylation and phosphorylation could be a useful tool to answer these questions [79].

Because of the easy handling, glutamine has been widely used as a mimic of acetylated lysine to study effects of acetylation on protein structures, functions, and interactions. However, the difference between the structures of glutamine and acetyllysine could be easily ignored. The length of glutamine side chain is about 6 Å, while that of acetyllysine is approximately 10.5 Å. Such difference could cause unexpected results [80, 81]. Indeed, in this study, we showed that the classic glutamine mutagenesis method and the genetic acetyllysine incorporation approach provided opposite results in some cases such as acetylation at K55 of ICDH (Figure 1b).

MATERIALS AND METHODS

General molecular biology

Chemical compounds in this study were purchased from Sigma-Aldrich, VWR International, or Chem-Impex International. DH5 α cells were used for general cloning. Polymerase chain reaction (PCR) reactions and plasmid constructions were performed by using the NEBuilder HiFi DNA Assembly Kit (New England Biolabs). Point mutations of the *icd* gene in the plasmid were made by the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). Inactivation of genes from the *E. coli* genome was performed by the recombination approach [82]. The strains and plasmids used in this study is listed in Table S1. CobB, YcgC, RutR, RcsB, and YfiQ proteins were expressed from the ASKA collection and purified by previous protocols [83].

Protein concentrations were measured by the Bradford Protein Assay (Bio-Rad). The purified ICDH and its variants were fractionated on a 4–20% SDS-PAGE gel and visualized by the Bio-Safe Coomassie Stain (Bio-Rad). For western blotting, the fractionated SDS-PAGE gel was transferred onto a PVDF membrane by using the Trans-Blot Turbo Transfer System (Bio-Rad). The PVDF membrane was incubated at room temperature with gentle shaking in the blocking buffer (5% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline) for 2 h. The horseradish peroxidase (HRP)-conjugated acetyllysine antibody (Cell Signaling Technology) was diluted 1:1000 with the blocking buffer and soaked the blocked PVDF membrane overnight at 4 °C. The membrane was prepared for chemiluminescence detection by using Pierce ECL Western Blotting substrates (Thermo Scientific).

Expression and purification of acetylated ICDH variants

The protocols followed previous studies with slightly modifications [54]. The genes of the ICDH and its variants were cloned into the *pCDF-1b* plasmid (EMD Millipore) with a C-terminal His₆-tag, and transformed into BL21 (DE3) cells (New England Biolabs) together with the *pTet* plasmids harboring the AcK incorporation system for expression [56, 84]. The expression strain was grown on 500 mL of LB medium supplemented with 100 μ g/mL

streptomycin and 50 µg/mL chloramphenicol at 37°C to an absorbance of 0.6–0.8 at 600 nm. Then protein expression was induced by the addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). For AcK-containing protein expression, media were additionally supplemented with 10 mM AcK and 20 mM nicotinamine (NAM). Cells were incubated at 25°C for an additional 12 h and harvested by centrifugation at 3200 × g for 15 min at 4 °C. The cell paste was suspended in 12 mL of lysis buffer [50 mM Tris (pH 7.8), 300 mM NaCl, 20 mM imidazole, 20 mM NAM, and 5 mM β-mercaptoethanol with cocktail protease inhibitors (Roche), and broken by sonication. The crude extract was centrifuged at 18,000 × g for 20 min at 4°C. The soluble fraction was filtered through a 0.45-µm membrane and loaded onto a column containing 2 mL of Ni-NTA resin (Qiagen) previously equilibrated with 20 mL lysis buffer. The column was then washed with 20 mL of washing buffer [50 mM Tris (pH 7.8), 300 mM NaCl, and 40 mM imidazole]. The protein bound to the column was finally eluted with 2 mL of elution buffer [50 mM Tris (pH 7.8), 300 mM NaCl, and 150 mM imidazole]. The purified protein was dialyzed with 2 L of storage buffer [25 mM Tris (pH 7.8), 10 mM NaCl, 1 mM dithiothreitol (DTT), and 50% glycerol], and stored at –80°C.

The ICDH enzyme assay and kinetic analyses

The enzyme activity assay followed the previous protocol [85]. The reaction was performed in the buffer containing 25 mM MOPS, 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT at 25°C. The reaction was carried out by mixing 30 ng ICDH or its variants, 1 mM isocitrate, and 1 mM NADP⁺ in a total volume of 100 µL. The rates of reaction were determined by monitoring the production of NADPH at 340 nm. For kinetic analyses, the concentration of one substrate was varied while the other substrate was at a fixed concentration of 5 mM. The kinetic parameters were calculated from nonlinear regression by software Grafit (Erithacus Software).

Mass spectrometry (MS) analyses

The full-length MS analyses were performed by the University of Arkansas Statewide Mass Spectrometry Facility, following the previous protocol [79]. Purified proteins were dialyzed with pure water, diluted to 0.05 mg/mL, and analyzed by a Bruker UltraflexII TOF-TOF with a MALDI ionization source. Spectra were obtained in the positive ion, linear mode. The LC-MS/MS analyses were performed by Yale University Keck Proteomics Facility, following previous protocols [86, 87]. Proteins were digested in gel by trypsin, and analyzed by LC-MS/MS on an LTQ Orbitrap XL equipped with a nanoACQUITY UPLC system. The Mascot search algorithm was used to search for the appropriate noncanonical substitution.

The *in vitro* acetylation assay

The acetylation assay was modified from previous experiments [83]. The reaction was performed in the buffer containing 50 mM Tris (pH 7.8), 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 10 mM sodium butyrate. The acetylation was carried out by mixing 10 µg ICDH, 10 µg of YfiQ, 0.2 mM acetyl-CoA in a total volume of 100 µL, and incubated at 37°C for 2 hours. For non-enzymatic acetylation, 10 µg ICDH was incubated with varying concentrations of acetyl-phosphate at 37°C for different time spans.

The *in vitro* deacetylation assay

The deacetylation reaction was slightly modified from the previous study [88]. The reaction was performed in buffer contains 40 mM HEPES (pH 7.0), 6 mM MgCl₂, 1.0 mM NAD⁺, 1 mM DTT and 10 % glycerol. The deacetylation was carried out by mixing 10 µg acetylated ICDH variants, 10 µg CobB or YcgC proteins in a total volume of 100 µL, and incubated at 37°C for 2 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ICDH	isocitrate dehydrogenase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
TCA	tricarboxylic acid
MDH	malate dehydrogenase
MS	mass spectrometry
AcK	N ^ε -acetyllysine
NAM	nicotinamine
AcP	acetyl-phosphate
ICL	isocitrate lyase

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Highlights

- The effect of acetylation on isocitrate dehydrogenase depends on positions.
- The acetylation level of isocitrate dehydrogenase is induced by glucose.
- CobB could deacetylate isocitrate dehydrogenase at specific positions.
- Isocitrate dehydrogenase could be acetylated without enzymes.

Figure 1. The effect of acetylation at single lysine residues

a) SDS-PAGE and western blotting analyses of purified ICDH and its variants. Lane 1, wild-type ICDH; lane 2, ICDH-55AcK; lane 3, ICDH-100AcK; lane 4, ICDH-142AcK; lane 5; ICDH-177AcK; lane 6, ICDH-230AcK; lane 7, ICDH-235AcK; lane 8, ICDH-242AcK; lane 9, ICDH-350AcK. The same amounts of proteins were loaded. b) Relative enzyme activities of ICDH and its variants with acetyllysine (AcK) or glutamine (Gln) substitution. The activity of wild-type *E. coli* ICDH was set as 1. Mean values and standard deviations were calculated based on three biological replicates.

Figure 2. The effect of simultaneous acetylation at two lysine residues

a) SDS-PAGE and western blotting analyses of purified ICDH and its variants. Lane 1, wild-type ICDH; lane 2, ICDH-55AcK/100AcK; lane 3, ICDH-55AcK/142AcK; lane 4, ICDH-55AcK/177AcK; lane 5, ICDH-55AcK/230AcK; lane 6, ICDH-55AcK/235AcK; lane 7, ICDH-55AcK/242AcK; lane 8, ICDH-55AcK/350AcK. The same amounts of proteins were loaded. b) Relative enzyme activities of ICDH and its variants with acetyllysine (AcK) at two lysine sites simultaneously. The activity of wild-type *E. coli* ICDH was set as 1. Mean values and standard deviations were calculated based on three biological replicates.

Figure 3. The effect of acetylation on ICDH *in vivo*

a) SDS-PAGE and western blotting of purified ICDHs from LB media (lane 1) and M9 media with 0.2% glucose (lane 2). The same amounts of proteins were loaded. b) The enzyme activities of purified ICDHs from LB media and M9 media with 0.2% glucose. The activity of ICDH purified from LB media was set as 1. The mean values and standard deviations were calculated based on three biological replicates.

Figure 4. Deacetylation of ICDH

a) SDS-PAGE and western blotting of purified ICDHs from wild-type BW25113 (lane 1), BW25113 *ycgC* (lane 2), and BW25113 *cobB* (lane 3). b) Western blotting of site-specifically acetylated ICDH variants treated with CobB for 2 hours. c) Western blotting of site-specifically acetylated ICDH variants treated with YcgC for 2 hours. The same amounts of proteins were loaded in all the experiments. d) Relative enzyme activities of ICDH and its acetylated variants after treatment with CobB or YcgC. The activity of wild-type *E. coli* ICDH without deacetylase treatment was set as 1. Mean values and standard deviations were calculated based on three biological replicates.

Figure 5. Acetylation of ICDH

a) SDS-PAGE and western blotting analyses of purified ICDHs from BW25113 wild-type (lane 1), *yfiQ* (lane 2), *cobB* (lane 3), and *yfiQ cobB* (lane 4). b) SDS-PAGE and western blotting analyses of purified ICDH treated with different concentrations of acetyl-phosphate (AcP). c) SDS-PAGE and western blotting analyses of purified ICDHs from wild-type BW25113 (lane 1), *pta* (lane 2), and *ackA* (lane 3). The same amounts of proteins were loaded in all the experiments.

Figure 6. Mapping of K55 and K350 on the ICDH structure

K55 and K350 were mapped on the structure of the ICDH dimer (PDB ID: 4aj3). Two subunits were colored with red and green separately. Only the side chains of labelled residues were shown.

Table 1

Kinetic analyses of ICDH and its acetylated variants

	k_{cat} (s^{-1})	$K_M, NADP^+$ (μM)	$K_M, isocitrate$ (μM)	$k_{cat}/K_M, NADP^+$ ($s^{-1} mM^{-1}$)	$k_{cat}/K_M, isocitrate$ ($s^{-1} mM^{-1}$)
ICDH WT	95.2 ± 3.1	15.9 ± 2.9	9.1 ± 1.4	5980	10400
ICDH 55AcK	123 ± 7.2	13.0 ± 1.7	9.5 ± 2.2	9460	12900
ICDH 100AcK	0.01 ± 0.00	278 ± 29	117 ± 34	0.036	0.086
ICDH 142AcK	104 ± 8.7	16.7 ± 1.5	9.0 ± 0.8	6220	11500
ICDH 177AcK	98.7 ± 4.0	15.6 ± 3.7	9.6 ± 1.0	6320	10300
ICDH 230AcK	0.24 ± 0.03	22.3 ± 3.1	376 ± 54	10.76	0.64
ICDH 235AcK	53.9 ± 2.6	18.9 ± 0.5	9.7 ± 1.8	2850	5560
ICDH 242AcK	90.7 ± 1.8	15.2 ± 1.2	8.9 ± 2.9	5960	10200
ICDH 350AcK	146 ± 25	9.2 ± 0.9	10.2 ± 0.5	15870	14300

The mean values and standard deviations were calculated based on three biological replicates. For K_M determination, one substrate was varied with six different concentrations, while the other substrate was fixed at a concentration of 5 mM. The parameters were determined by nonlinear regression with software GraFit (Erithacus Software).