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Acetylation, ADP-ribosylation and methylation of malate dehydrogenase

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Abstract

Metabolism within an organism is regulated by various processes, including post-translational modifications (PTMs). These types of chemical modifications alter the molecular, biochemical, and cellular properties of proteins and allow the organism to respond quickly to different environments, energy states, and stresses. Malate dehydrogenase (MDH) is a metabolic enzyme that is conserved in all domains of life and is extensively modified post-translationally. Due to the central role of MDH, its modification can alter metabolic flux, including the Krebs cycle, glycolysis, and lipid and amino acid metabolism. Despite the importance of both MDH and its extensively post-translationally modified landscape, comprehensive characterization of MDH PTMs, and their effects on MDH structure, function, and metabolic flux remains underexplored. Here, we review three types of MDH PTMs – acetylation, ADP-ribosylation, and methylation – and explore what is known in the literature and how these PTMs potentially affect the 3D structure, enzymatic activity, and interactome of MDH. Finally, we briefly discuss the potential involvement of PTMs in the dynamics of metabolons that include MDH.

General overview of PTMs and malate dehydrogenase

Post-translational modifications (PTMs) are covalent alterations of amino acids in a peptide chain. The dynamics of PTMs rely on either non-enzymatic or enzymatic mechanisms, with a ‘writer’ that adds a specific PTM, and an ‘eraser’ that removes it. These PTMs can be recognized by ‘readers’ which are proteins with specialized domains that recognize and bind to post-translationally modified proteins to mediate specific cellular outcomes. While protein expression levels may not change, protein function and behavior may be altered as a result of PTMs on various protein isoforms in different cellular compartments.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

More than 400 different PTMs have been identified, such as phosphorylation, acetylation, ADP-ribosylation, and methylation, which differentially modulate the charge, structure, activity, location, and molecular interactions of a protein [1]. Histones are DNA-binding proteins that are components of chromatin which undergo extensive PTM and have been shown to contribute to epigenetic mechanisms involved in diseases like cancers [2]. The extensive study of chromatin regulation by PTMs has led to the recognition of a 'histone code' which is 'read' by specialized proteins that interact with histones and other proteins to render the chromatin either more or less accessible, for the modulation of DNA replication, gene expression, and the DNA damage response. This elaborate system is difficult to characterize. For example, the choice between gene expression and silencing is dependent on the specific amino acid within the histone protein sequence that is methylated [3]. Moreover, the quantity, duration, and location of the PTM on proteins have importance, and the combination of PTMs directly balances the equilibrium between healthy and disease states [4]. While the precise mechanisms involving writing and reading the histone code are still not perfectly understood, these mechanisms have been extensively studied and can serve as a model for other cellular processes in which enzymes are regulated by PTM. Enzymes involved in metabolic pathways, for example, have been shown to be abundantly post-translationally modified, which provides a mechanism to finely tune their activity to rapidly respond to changes in the surrounding environment. However, the characterization of these PTMs on metabolic enzymes has not, so far, led to the establishment of an analogous 'metabolic code'. Despite this lack of code, increasing evidence suggests a link between the PTMs on hundreds of metabolic enzymes, metabolic modulation, and diseases [5,6].

Malate dehydrogenase (MDH) is an essential metabolic enzyme, expressed from different genes and localized in various cellular compartments, such as the cytosol, mitochondria, chloroplasts, and peroxisomes (Figure 1) [7,8]. All MDH isoforms catalyze the reversible interconversion of malate and $NAD(P)^+$ to oxaloacetate and NAD(P)H. The roles of MDH vary widely from producing cellular energy via the Krebs cycle to maintaining redox balance, energy flux between cellular compartments, and the cross-talk between metabolic pathways via the malate-aspartate shuttle [7,8]. Physiologically, the directionality of the reaction is regulated by the presence of various metabolites and the energetic needs of the cell [9]. Structurally, MDH is a homodimer or homotetramer and is subjected to extensive allosteric regulation through protein-protein interactions and by small molecule binding such as L-malate, oxaloacetate, citrate, glutamate, and α-ketoglutarate. In addition to fundamental studies of the biochemical, structural, and functional relationships of various MDH isoforms, this protein has been widely analyzed from a physiological perspective for its roles in metabolic disorders, neurodegenerative diseases, and cancer [10,11].

The MDH enzyme is substantially modified through a variety of PTMs, including phosphorylation, acylation, ADP-ribosylation, and methylation. However, the roles of these PTMs on MDH structure, function, and cellular physiology are complex. One challenge to studying the biochemical and cellular implications of PTMs on metabolic enzymes is that the precise function of a PTM may differ based on the organism or tissue in which it is analyzed. Moreover, the mere presence of a PTM at a site on a protein does not necessarily confer a specific functional or regulatory alteration. In this review, we discuss examples of MDH acylation, ADP-ribosylation, and methylation PTMs and their consequences on

metabolism, when known. We conclude by briefly reviewing the potential role of MDH PTMs in the integrity of metabolons. A review of the impacts of MDH phosphorylation is described elsewhere (covered in this issue).

MDH acetylation and acylation

Acetylation is one of the most abundant PTMs identified on proteins [12]. This modification reduces the overall electrostatic positive charge of the amino group, either on the *N*-α-amino group of the *N*-terminus or the *N*-ε-amino group of a lysine side chain (Figure 2A). While *N*-α-acetylation is more common, here we will focus on the latter modification. *N*-ε-acetylation (AcK) PTM occurs via both non-enzymatic and enzymatic mechanisms. Non-enzymatic protein acetylation occurs using a reactive acyl donor such as acetyl phosphate (AcP) or acetyl CoA (AcCoA) [13,14]. In contrast, enzymatic acetylation is mediated by *N*-acetyltransferases that require AcCoA as a substrate [13,14]. The enzymes that acetylate lysine residues specifically are referred to as lysine (K) acetyltransferases (KATs) and typically belong to the Gcn5-related *N*-acetyltransferase (GNAT), MYST, and p300/CBP protein families [15]. Some acetyl groups can be removed by deacetylase enzymes, such as CobB in *Escherichia coli*, and SIRT3 in eukaryotic systems [16-18]. Yet, it is currently unclear how these enzymatic and non-enzymatic acetylation/deacetylation mechanisms affect the stoichiometry of protein acetylation and their influence and impact on protein function [19]. Hence, the reversible nature of acetylation makes it an effective strategy for rapidly modulating protein charge, solubility, structure, and interactions with other biomolecules. *N*-ε-AcK acetylation as a response to a variety of growth conditions and stimuli is a conserved PTM across the domains of life [12,13]. Acetylated proteins from eukaryotic to prokaryotic systems have been observed, but not all acetylation sites on all proteins are conserved across different organisms or isoforms [20]. Furthermore, the functional importance of AcK residues, when known, is not necessarily identical.

MDH acetylation in bacteria

Several studies have investigated *E. coli* MDH (EcMDH) acetylation *in vitro* and *in vivo*. *In vivo*, AcP and/or AcCoA acetylation of EcMDH has been described on K99, K140, K272, and K301 (Figure 2B,C) [21-23]. These studies indicate that MDH acetylation in this bacterium is strictly non-enzymatic. Functional analysis using site-specific AcK incorporation on AcK99 and AcK140 in EcMDH showed acetylation increases enzyme turnover without changing the apparent affinity for either substrate, malate, or NAD^+ [23,24]. Acetylation of K140 on *Vibrio cholerae* MDH has also been observed and corresponds to the EcMDH K140 residue [25]. Finally, acetylation of EcMDH has been detected in the presence of glucose, but at a lower level in citrate-containing media [22,26]. This observation suggests that in *E. coli* MDH acetylation is dependent upon the energy state of the cell.

MDH acetylation in humans

Unlike bacteria, where only one MDH is present, multiple MDH isoforms are found in different cellular compartments in higher organisms. In humans, two isoforms hMDH1 and hMDH2 are located in the cytosol and mitochondria, respectively. Numerous AcK

residues have been identified for human MDH isoforms in different cell types (Figure 2B,C) [23,27-32]. For example, hMDH1 and hMDH2 were shown to be acetylated in acute myeloid leukemia MV4-11 cells on K118 and K298 for hMDH1, and K165, K185, K301, K329, and K335 for hMDH2 [27]. In Chang liver cells, acetylation was identified on K185, K301, K307, and K314 of hMDH2. Acetylation was increased in these cells in the presence of a high glucose concentration and the acetylated hMDH2 enzyme showed higher activity [32]. However, only hMDH2 AcK307 was shown to alter enzyme turnover, without changing the apparent affinity for the malate and NAD^+, similar to AcK99 and AcK140 in EcMDH [23,24]. While numerous studies have shown that acetylation level in the nucleus has a direct effect on metabolism reprogramming and tumorigenesis, the direct contribution of MDH acetylation in these processes has not been specifically addressed [33].

The physiological roles of acetylated hMDH1 and hMDH2 lysine residues have been examined in the context of adipogenesis and caloric restriction, respectively. For example, hMDH1 is overexpressed and acetylated on K118 during adipogenesis and adipocyte differentiation [30]. Acetylated hMDH1 shows increased activity, generating malate in the cytosol that will be used by the citrate shuttle, a metabolic pathway that produces AcCoA and NADPH required for fatty acid synthesis (Figure 3). In response to caloric restriction, hMDH2 K239 is deacetylated in mouse liver, but is acetylated in the absence of the deacetylase SIRT3 [28]. This residue is located at the dimer interface, and its mutation to glutamine, an uncharged polar amino acid used to crudely mimic acetylated lysine, decreases enzymatic activity, which may suggest a role for acetylation in the stability of the hMDH2 quaternary structure [34]. This same study showed that other enzymes involved in the Krebs cycle, including citrate synthase (CS), aconitase, and succinate dehydrogenase, are acetylated in response to SIRT3 loss, affecting their enzymatic activity and the metabolite profile [28]. Altogether, the alteration of acetylation dynamics is associated with a rewiring of mitochondrial-dependent metabolism [28].

MDH acetylation in plants

Like humans, plants have multiple MDH isoforms in various cellular compartments. In *Arabidopsis thaliana* (At), at least eight NAD^+-dependent MDH isoforms have been observed in the cytosol, mitochondria, chloroplasts, and peroxisomes [35,36]. However, each isoform displays unique characteristics, including substrate specificity, tissue expression, and PTM profile. For instance, the mitochondrial isoform AtmMDH1 is more widely expressed than AtmMDH2 [37]. Lysine acetylation has been identified on AtmMDH1 (K170, K325, K329, and K334), AtmMDH2 (K170), peroxisomal isoform AtpMDH1 (K182), and cytosolic AtMDH2 (K3) (Figure 2B,C) [29,31,38,39].

The consequences of acetylation have been further studied in AtmMDH1 to determine kinetic parameters in both reaction directions [38]. The site-specific incorporation of AcK325 and AcK329 did not reveal changes in MDH kinetic parameters for oxaloacetate but decreased enzyme turnover was observed for AcK170 and AcK334 *in vitro*. On the other hand, AcK325, AcK329, and AcK334 were associated with an increased apparent affinity for malate and decreased turnover number, while AcK170 reduced the apparent affinity for malate. Similar effects were observed in another plant model, *Physcomitrella patens* [38].

Consequently, acetylated lysine residues may play a role in the directionality of the reaction mediated by MDH, impacting metabolic flux. The widespread occurrence of this PTM in plants highlights the relevance of acetylation in modulating the structure and function of MDH (for a review, see [33]).

Other MDH acylation modifications

Acylation involves the transfer of an acyl chain from an acyl CoA donor molecule to a lysine or cysteine residue [40]. The diversity of acyl groups that can decorate protein residues increases the possibility of finely tuning the charge, hydrophobicity, and regulation of a particular protein. Depending on the identity of the acyl group, the charge of a lysine residue can vary from +1 to neutral for an acetyl group or from +1 to −1 for succinyl or malonyl groups under physiological conditions (Figure 2A) [41]. In addition to acetylation, both hMDH1 and hMDH2 have been found to be succinylated and malonylated on lysine residues, including succinylation of hMDH1 K298, malonylation of hMDH2 K185 and K301, and both succinylation and malonylation of hMDH2 K301 and K329 (Table 1) [29,31].

Like acetylation, the transfer of succinyl or malonyl groups from succinyl CoA and malonyl CoA, respectively, to the *N*-ε-amino group of a lysine residue can occur by enzymatic or non-enzymatic mechanisms [42,43]. KAT2A (or hGCN5) has been identified as both a succinyltransferase and malonyltransferase [44-46]. The removal of these PTMs is mediated by Sirtuin proteins, especially SIRT5 and SIRT7 [47,48]. Currently, these PTMs remain less well-understood than acetylation. However, processes that contribute to the synthesis and degradation of these acyl CoA substrates seem to play an important role, such as altering malonylation levels on proteins in the absence of malonyl CoA synthetase or malonyl CoA decarboxylase [49,50]. Furthermore, both succinylation and malonylation have been described on numerous metabolic enzymes and the loss of SIRT5 is associated with hyper-succinylation and hyper-malonylation of mitochondrial proteins [47,51,52]. Current literature suggests that these PTMs contribute to the overall modulation of carbohydrate and fatty acid metabolism, and they are dysregulated in diseases like diabetes and cancer [52-54]. Understanding what stimulus would lead to different types of acylation and the outcomes associated with each PTM remain important challenges for the future.

MDH ADP-ribosylation

ADP-ribosylation is mediated by ADP-ribosyltransferases, which transfer ADP-ribose from NAD^+ onto an amino acid side chain (currently nine are known, including glutamate and serine) [55]. ADP-ribosylation can form *N*-, *O*-, or *S*-glycosidic linkages between the ribose of the ADP-ribose and the side chain of the targeted amino acid [56]. In humans, several members of the poly(ADP-ribose) polymerase protein (PARP) family, possess ADP-ribosyltransferase activity [57]. The biological meaning of this PTM is complex and dependent upon the modified amino acids and the number of ADP-ribose moieties that are added (Figure 4A) [55]. For instance, PARP10 transfers one ADP-ribose moiety in a mono-ADP-ribosylation reaction, whereas PARP1 and PARP2 can add multiple ADP-ribose moieties to form a branched polymer in a poly-ADP-ribosylation process. The dynamics

of this reaction are mediated by both PARG (poly(ADP-ribose) glycohydrolase) and ARH3 (ADP-ribosylhydrolase), which trim poly(ADP-ribose) chains or remove ADP-ribose moieties from the amino acid without generating NAD^+ [58].

The development of mass spectrometry methods to identify ADP-ribosylated proteins has led to the discovery of new PARP targets [59-61]. These targets include numerous proteins involved in metabolism, including hMDH1 and hMDH2 [62-64]. Both isoforms are ADP-ribosylated: S241 and E329 for hMDH1 and E84 and S317 for hMDH2 (Figure 4B,C), but the functional consequences of these modifications are unknown. Yet, their functional meaning may be discerned by considering the location of the ADP-ribosylated residues. The human MDH1 S241 residue is known to be involved in NAD^+/NADH binding and is close to the dimerization interface where it interacts with amino acids involved in stabilizing the quaternary structure (e.g. Q238) [65,66]. Thus, S241 ADP-ribosylation may affect enzymatic activity by blocking access to the substrate binding pocket and/or dimerization. Due to its surface exposed location, E329 ADP-ribosylation may have a potential role in protein-protein interactions with molecules containing ADP-ribose binding motifs.

A similar impact of solvent-exposed hMDH2 ADP-ribosylated residues E84 and S317 may contribute to the interaction with proteins specifically recognizing and binding to the ADP-ribose modification. Interestingly, the hMDH1 S241 and hMDH2 S317 residues have also been shown to be phosphorylated, which suggests a potential competition between ADP-ribosylation and phosphorylation [67]. Cross-talk between these two PTMs has already been observed to differentially modulate protein functions [68-73].

MDH methylation

Once thought to be a modification exclusive to histones, it is now well known that lysine and arginine residues of numerous proteins are substrates for *S*-adenosylmethionine (AdoMet)-dependent methyltransferases, such as AdoMet-protein arginine methyltransferases (PRMTs) [74]. Unlike lysine, arginine methylation maintains the side chain positive charge (Figure 5A). Additionally, while lysine residues can only be modified by a single acyl group, arginine residues can be mono- or di-methylated. Di-methylated arginine residues can be asymmetrical (two methyl groups on a single nitrogen) or symmetrical (two nitrogen atoms each with a single methyl group). A wide variety of methods have been developed to identify methylated proteins with an array of antibodies specific for mono-, symmetrical di-, and asymmetrical dimethylated arginine [75,76]. In addition to immunological methods, ion exchange techniques can be used to identify methylated arginine residues; these techniques can be used together for enhanced identification.

To date, no methylation sites have been identified on EcMDH or AtMDH. In contrast, both hMDH1 and hMDH2 isoforms are methylated (Figure 5B,C). Cytosolic MDH1 is asymmetrically di-methylated on R230 located at the dimer interface [77,78]. The protein methyltransferase CARM1/PRMT4 catalyzes methylation of this arginine, which inhibits MDH1 activity by disrupting the ability of the enzyme to dimerize. By altering the quaternary structure, this CARM1-dependent methylation diminishes flux through the malate-aspartate shuttle to reduce the level of mitochondrial respiration. This modification

also seems associated with the cellular stress level, as CARM1 activity is reduced in response to oxidative stress. Human MDH1 has also been found monomethylated on K110 by SMYD2, though the functional significance of this modification is unknown [79]. It is noteworthy that CARM1 and SMYD2 have been shown to be overexpressed in cancers [79]. To date, methylation of mitochondrial hMDH2 R257 has been shown in two methyl-proteomics studies [80,81]. However, the functional relevance of this particular modification has not been investigated. While PRMT inhibitors have been used in the proteomic study, the identity of the specific methyltransferase(s) responsible for this MDH methylation remains elusive [81].

Potential PTM role in the organization of metabolon

Substantial evidence supports the existence of an *in vivo* metabolon including Krebs cycle proteins MDH2 and CS, as well as aconitase, succinyl CoA synthetase (SCS), fumarase, and isocitrate dehydrogenase (IDH), in eukaryotes and bacteria [82-84]. Co-localization of multiple enzymes involved in a metabolic pathway offers certain advantages such as channeling of metabolic intermediates directly from one active site to another and provides an opportunity for regulation [85-87]. Recent studies show that MDH2 and CS share a common interface [84,85]. Interestingly, that interface includes hMDH2 E84, which, as noted above, can be ADP-ribosylated. Work defining the MDH2-CS interface also identified K301 of MDH2 through a chemical cross-link which may indicate that this acetylatable residue is involved in the interaction with CS. As in the case of hMDH1 R230me2a which inactivates the enzyme by disrupting subunit-subunit interactions, other PTMs may offer a similar regulatory function by affecting metabolon assembly.

A multiprotein complex in the fungus *Cunninghamella bainieri* involved in lipid biosynthesis, has been identified and is composed of MDH1, malic enzyme (ME), fatty acid synthase (FAS), ATP:citrate lyase (ACL), acetyl CoA carboxylase (ACC), pyruvate carboxylase (PC), and MDH [88]. Further characterizations are needed to understand how this metabolon is organized and regulated and whether it is conserved across species. Thus, unveiling the roles of PTMs on MDH and other metabolic enzymes like CS will provide a better understanding of the dynamics of metabolons in response to environmental conditions.

Future perspectives

The development of suitable tools, such as mass-spectrometry methods, has unveiled a large repertoire of protein PTMs, including acylation, ADP-ribosylation, and methylation [40,60,75]. Yet, identifying their functional implications, if any, is still challenging. For each PTM, the environmental conditions and the enzymes involved in the PTM dynamics need to be determined to understand the impacts on the enzymatic activity, three-dimensional structure, subcellular localization, and interactome (Figure 6). By cross-listing PTMs on MDH, a comprehensive picture of how MDH modulates metabolism and contributes to diseases will be established.

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Abbreviations

ACC	acetyl CoA carboxylase
AcCoA	acetyl CoA
AcK	acetylated Nε-amine of a lysine
ACL	ATP:citrate lyase
AcP	acetyl phosphate
AdoMet	S-adenosylmethionine
ARH	ADP-ribosylhydrolase
CS	citrate synthase
FAS	fatty acid synthase
GNAT	Gcn5-related *N*-acetyltransferase
IDH	isocitrate dehydrogenase
KAT	lysine (K) acetyltransferase
MDH	malate dehydrogenase
ME	malic enzyme
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PARG	poly(ADP-ribose)glycohydrolase
PARP	poly(ADP-ribose)polymerase
PC	pyruvate carboxylase
PRMT	AdoMet-protein arginine methyltransferase
PTM	post-translational modification
SCS	succinyl CoA synthetase
SIRT	sirtuin

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Summary

- MDH is extensively modified by acylation, ADP-ribosylation, and methylation.
- The role of these PTMs remains mostly uncharacterized, but growing evidence indicates their contribution in modulating MDH structure, catalytic activity, and interactome.
- Further study of the metabolon will provide an understanding of the importance of PTMs in the stability and regulation of this multiprotein complex.

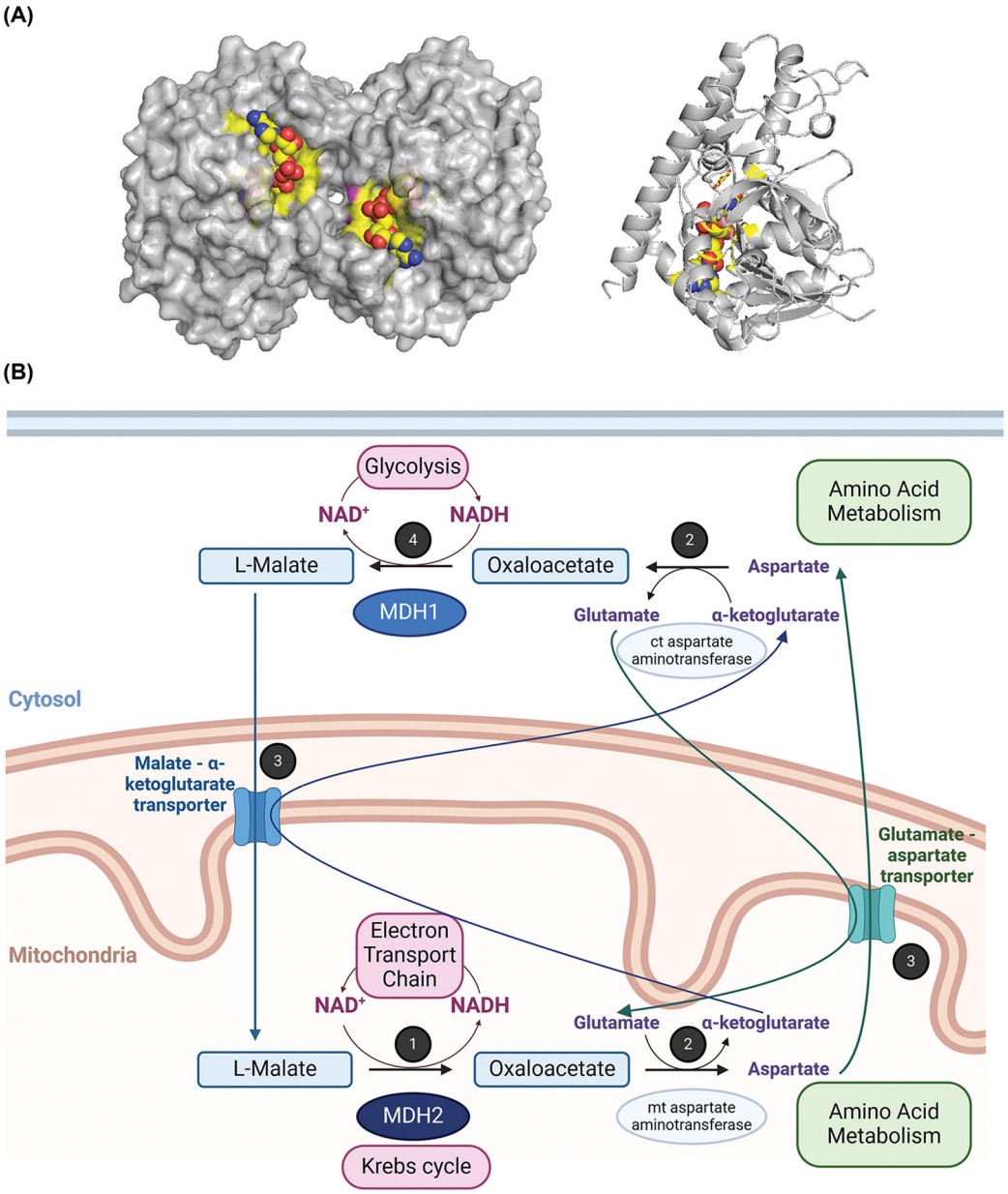

Figure 1. Structure and roles of human MDH in the metabolism

(**A**) Three-dimensional structures of porcine cytoplasmic MDH dimer (surface view) and left monomer (ribbon view) are represented (PDB: 5mdh). The active site is highlighted in yellow, and the NAD^+ ligand is shown in yellow spheres. Monomeric MDH is presented in the same orientation in Figures 2-4, with the active site facing back compared to the dimeric representation. (**B**) Overview of the human MDH roles and link to energy production by the electron transport chain, redox balance, and cross-talk between metabolic pathways. 1: Reaction 8 of the Krebs cycle: Oxidation of malate associated with the reduction of NAD^+ by mitochondrial MDH2. NAD^+ is regenerated via the electron transport chain. 2: A transamination reaction links the malate shuttle to amino acid metabolism. 3: Conversion of oxaloacetate to malate in the malate-aspartate shuttle requires the assistance of the

glutamate-aspartate antiporter and the malate-α-ketoglutarate antiporter. 4: Reduction of oxaloacetate with concomitant oxidation of NADH by cytosolic MDH1. NAD^+ is used by metabolic pathways like glycolysis. This figure was generated using PyMOL and BioRender.com.

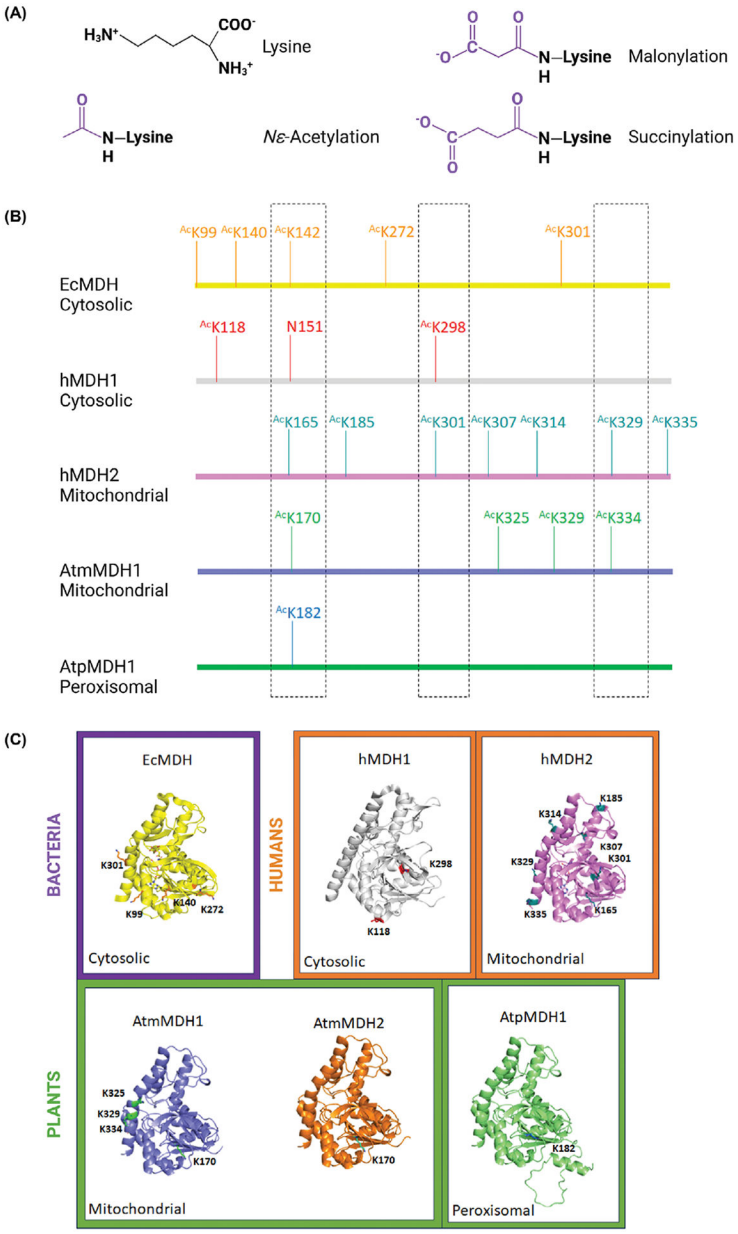

Figure 2. Sequence and structural comparison of acetylated lysine residues identified on proteins from different domains of life

(**A**) Chemical structures of known lysine acylations. (**B**) A cartoon representation of locations of acetylated lysine residues on the linear amino acid sequences based on the structural alignment of multiple MDH isoforms from different organisms. (**C**) Three-dimensional structure of multiple monomeric MDH isoforms from different organisms. Bacterial proteins are surrounded by a purple box, human proteins with orange boxes, and plants with green boxes. The cellular localization is noted (i.e., cytosolic, mitochondrial, and peroxisomal). The crystal structure of the *E. coli* MDH enzyme (PDB: 1emd; UniProtID: P61889) is shown in yellow ribbons; citrate and NAD are in yellow sticks. Lysine residues identified as acetylated are in orange sticks. The crystal structure of the human MDH1 enzyme (PDB: 7rm9; UniProtID: P40925) is shown in grey ribbons, with identified AcK

residues in red sticks. The crystal structure of the human MDH2 enzyme (PDB: 4wlo; UniProtID: P40926) is shown in pink ribbons; oxaloacetate and NADH are in pink sticks, and identified AcK residues are in teal sticks. AlphaFold models for *A. thaliana* proteins with identified AcK residues are represented in purple ribbons and green sticks for AtmMDH1 (UniProtID: Q9ZP06), in orange ribbons with cyan sticks for AtmMDH2 (UniProtID: Q9LKA3) and in green ribbons with blue sticks for peroxisomal AtpMDH1 (UniProtID: O82399). The cartoon sequence alignment shows conserved residues in the three-dimensional space with dashed outlined boxes. Not all residues are included. Three-dimensional structures were generated using PyMOL and the figure was constructed using BioRender.com.

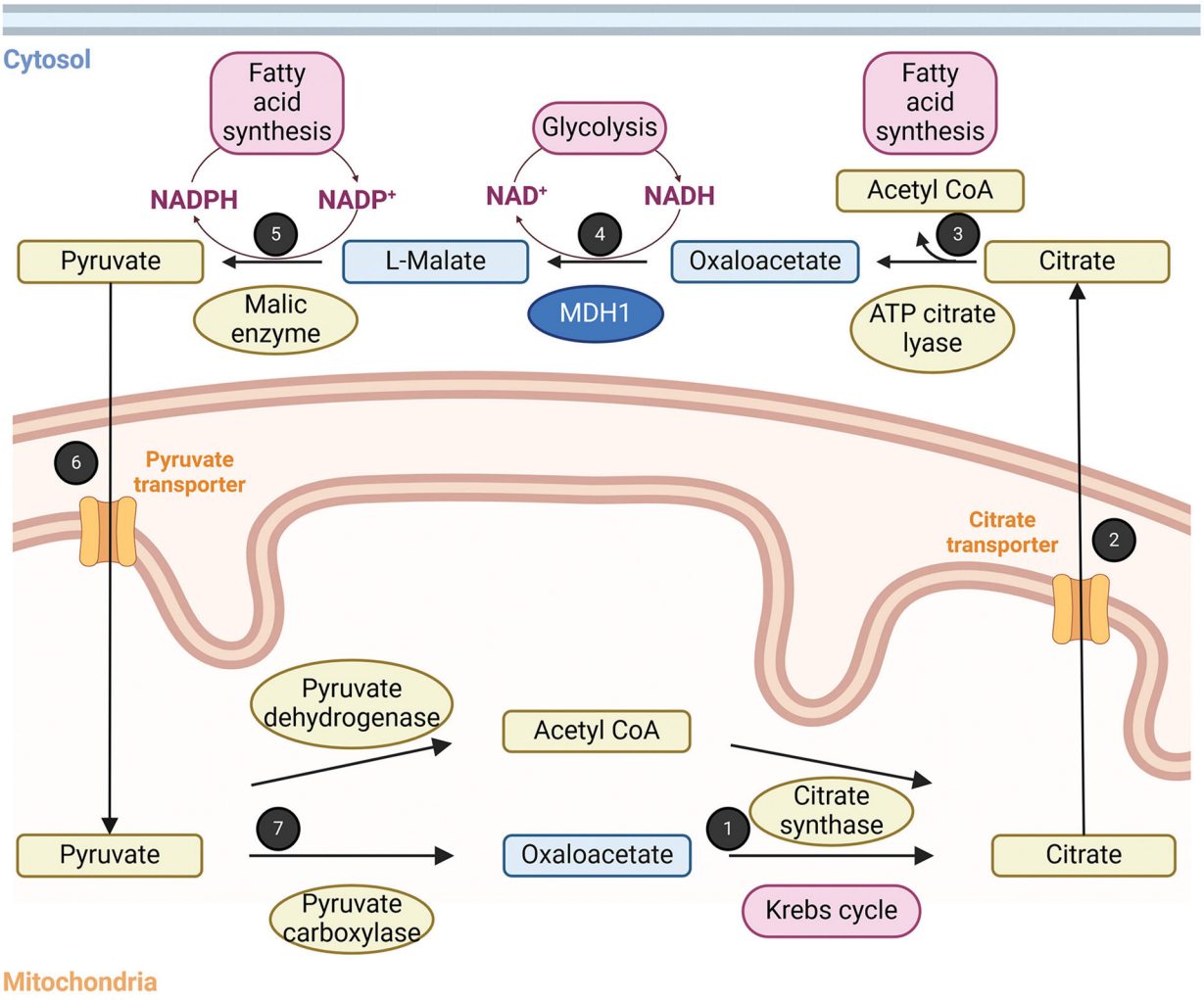

Figure 3. Role of MDH1 in the citrate shuttle and in fatty acid synthesis

1: Reaction 1 of the Krebs cycle: Condensation of acetyl unit from the acetyl CoA with oxaloacetate by citrate synthase. 2: Transport of citrate from the mitochondria to the cytosol by the citrate transporter. 3. Cleavage of citrate to acetyl CoA and oxaloacetate by ATP citrate lyase. Acetyl CoA can be used as a precursor of fatty acids. 4: Reduction of oxaloacetate with concomitant oxidation of NADH by cytosolic MDH1. NAD^+ is used by metabolic pathways like glycolysis. 5: Oxidative decarboxylation of malate by the $NADP^+$-dependent malic enzyme to generate pyruvate. The generated NADPH is used for fatty acid synthesis. 6: Transport of the pyruvate from the cytosol to the mitochondria by the pyruvate transporter. 7: Pyruvate is used to form oxaloacetate via a carboxylation reaction by pyruvate carboxylase, and acetyl CoA by an oxidative decarboxylation reaction by pyruvate dehydrogenase. This figure was generated with BioRender.com.

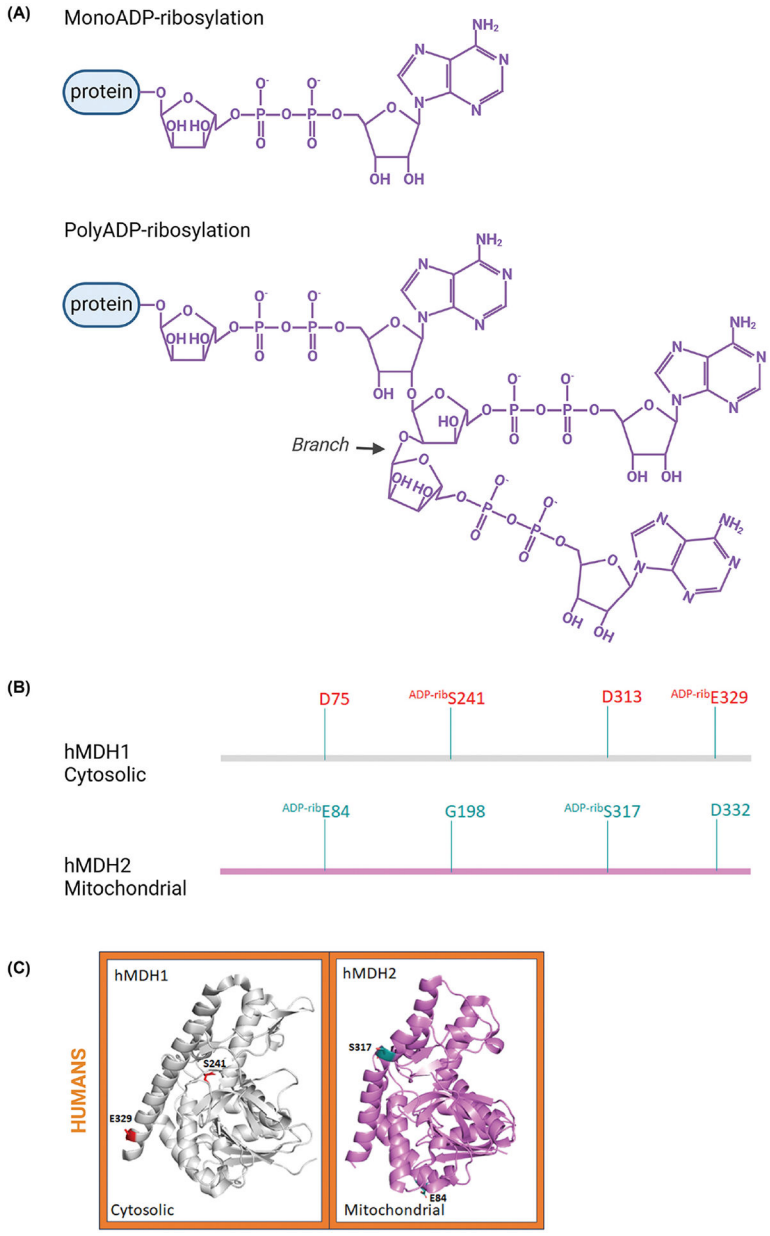

Figure 4. Sequence and structural comparison of ADP-ribosylated (ADP-rib) residues identified on human MDH proteins

(**A**) Chemical structures of mono- and poly-ADPribose. (**B**) A cartoon representation of locations of ADP-ribosylated residues on the linear amino acid sequences based on the structural alignment of human MDH isoforms. (**C**) The three-dimensional structure of monomeric human MDH1 (PDB: 7rm9; UniProtID: P40925) and MDH2 (PDB: 4wlo; UniProtID: P40926) isoforms with ADP-ribosylated residues. These structures are colored as described in Figure 2. Three-dimensional structures were generated using PyMOL and the figure was constructed using BioRender.com.

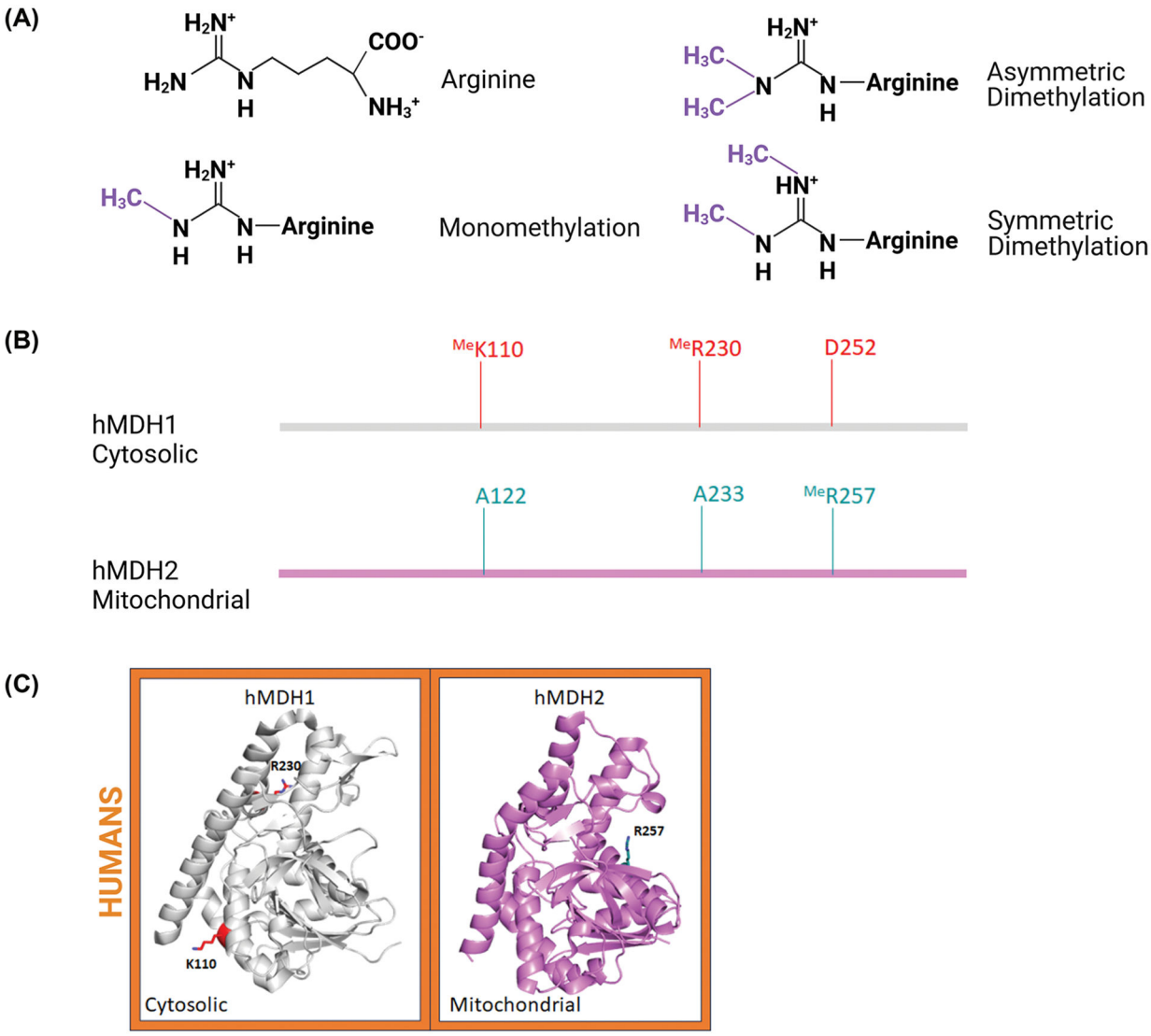

Figure 5. Sequence and structural comparison of methylated (Me) residues identified on human MDH proteins

(**A**) Chemical structures of known arginine methylations. (**B**) Methylated residues represented on the linear sequence of human MDH isoforms. (**C**) Crystal structure of monomeric human MDH1 (PDB: 7rm9; UniProtID: P40925) and MDH2 (PDB: 4wlo; UniProtID: P40926) isoforms with methylated residues. These structures are colored as described in Figure 2. Three-dimensional structures were generated using PyMOL and the figure was constructed using BioRender.com.

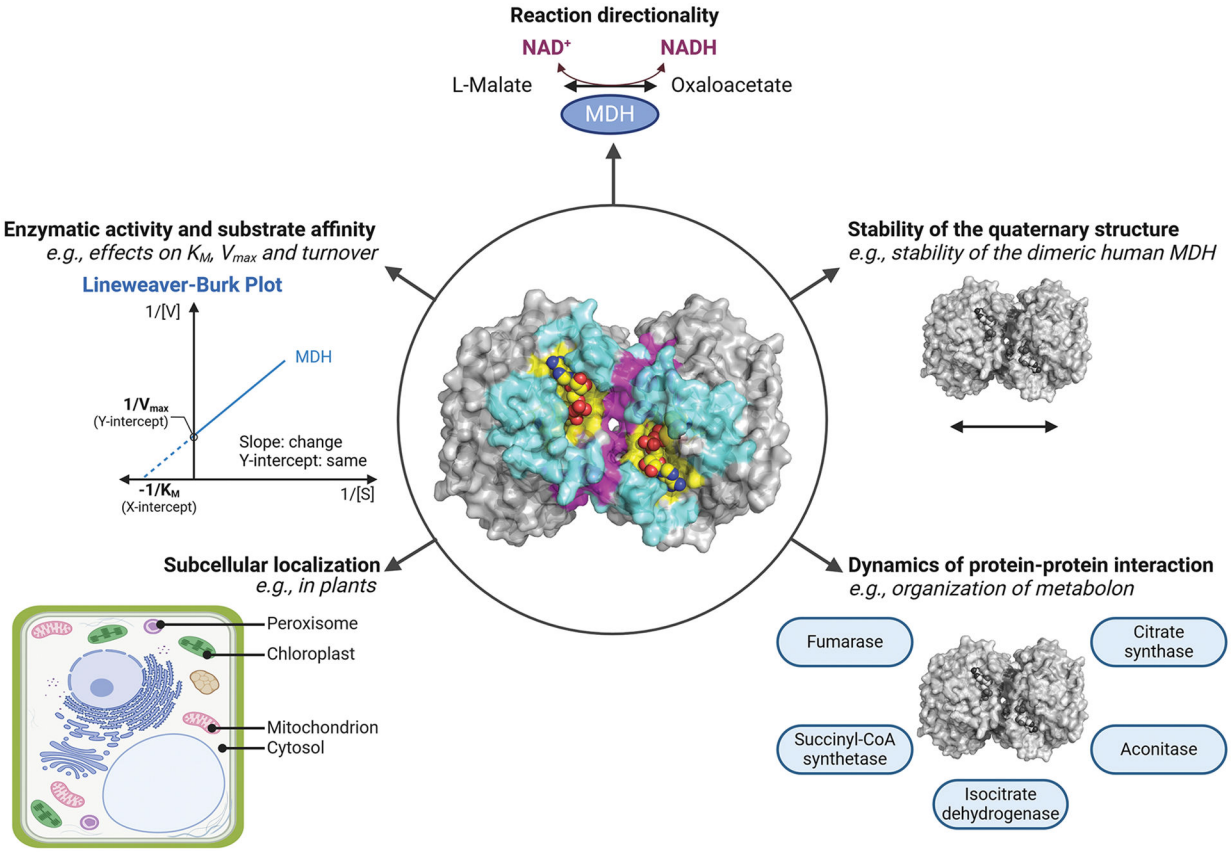

Figure 6. Potential roles of PTMs on MDH

At the center, the three-dimensional structure of the porcine cytoplasmic MDH dimer is represented (PDB: 5mdh). The active site is highlighted in yellow, the dimer interface in magenta, and the citrate synthase surface for the metabolon complex in cyan. The NAD^+ ligand is shown in yellow spheres. Properties that can be modulated by PTMs, including defining the directionality of the reaction, altering enzymatic characteristics (e.g. K_m and V_{max}), defining the subcellular localization, regulating the stability of the quaternary structure and protein–protein interactions protrude around the circle. This figure was generated using PyMOL and BioRender.com.

Table 1

List of identified succinylated and/or malonylated lysine residues on human MDH that are known to also be acetylated

Modification	hMDH1	hMDH2
Succinylation	K103, K107, K110, K214, K298	K91, K157, K203, K296, K324, K329, K335
Malonylation	K110	K78, K185, K203, K239, K296, K301, K307, K314, K324