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Novel roles of holocarboxylase synthetase in gene regulation and intermediary metabolism

Janos Zempleni, Dandan Liu, Daniel Teixeira Camara, and Elizabeth L. Cordonier

Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, Nebraska, USA.

Abstract

The role of HLCS in catalyzing of covalent binding of biotin to the 5 human biotin dependent carboxylases is well established, as are the essential roles of these carboxylases in the metabolism of fatty acids, catabolism of leucine, and gluconeogenesis. Here we review recent discoveries regarding the roles of HLCS in assembling a multiprotein gene repression complex in chromatin. We also discuss emerging evidence suggesting that the number of biotinylated proteins is far larger than previously assumed, including members of the heat shock superfamily of proteins and proteins coded by the *ENO1* gene. Evidence is presented linking biotinylation of heat shock proteins HSP60 and HSP72 with redox biology and immune function, respectively, and biotinylation of the two *ENO1* gene products MBP-1 and ENO1 with tumor suppression and glycolysis, respectively.

Keywords

chromatin; epigenetics; gene regulation; holocarboxylase synthetase; metabolism

Introduction

Holocarboxylase synthetase (HLCS) is a monomeric protein biotin ligase, encoded by a single gene localized on chromosome 21 (21q22.13) in the human genome.^{1, 2} Its expression is regulated by three promoters, denoted P1, P2, and P3.^{3, 4} The promoters lack the TATA box that is typical for housekeeping genes. Promoter activities follow the pattern P1>>P3>P2 in various human cell lines. Human full-length HLCS comprises 726 amino acids with a predicted molecular weight of 81 kDa.⁵ Three HLCS transcripts plus additional splicing variants originate in exons 1, 2, and 3 of the gene⁵; methionines 1, 7, and 58 in exons 6 and 7 have been identified as possible translation start sites.⁶ HLCS proteins, migrating with apparent sizes of 62, 64, 76, 82, and 86 kDa during gel electrophoresis, have been detected in human placenta and in bovine liver by using anti-HLCS. The 76-kDa, 82 kDa, and 86 kDa bands probably represent HLCS with translation start sites in methionines

Correspondence: Janos Zempleni, Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, 316C Leverton Hall, Lincoln, Nebraska 68583-0806, USA. jzempleni2@unl.edu. Phone: +1-402-472-3270. Fax: +1-402-472-1587..

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58, 7, and 1, respectively, whereas bands of lower molecular weight might have been caused by protein degradation.^{1, 6}

Biotinylation of proteins by HLCS depends on ATP and proceeds in the following two steps.⁷⁻⁹ In all known HLCS-dependent reactions, biotin is attached covalently to the ϵ -amino group of a lysine (K) residue.¹⁰

(1) $\text{ATP} + \text{biotin} + \text{HLCS} \rightarrow \text{Biotinyl-5'-AMP-HLCS} + \text{pyrophosphate}$

(2) $\text{Biotinyl-5'-AMP-HLCS} + \text{apo-protein} \rightarrow \text{holoprotein} + \text{AMP} + \text{HLCS}$

(Net) $\text{ATP} + \text{biotin} + \text{apoprotein} \rightarrow \text{holoprotein} + \text{AMP} + \text{pyrophosphate}$

The “classical” role of holocarboxylase synthetase is that as the enzyme that catalyzes the attachment of biotin to five carboxylases in humans (Figure 1).¹⁰ The sequence of amino acids in the region of the biotinylated lysyl residue of the carboxylases (AMKM) is conserved in different species.¹¹

(a) The human proteome contains two acetyl-CoA carboxylases — ACC1 and ACC2.¹² Both catalyze the covalent binding of bicarbonate to acetyl-CoA, thereby producing malonyl-CoA, a key intermediate in fatty acid metabolism. Acetyl-CoA carboxylase 1 is a 265-kDa protein and localizes in the cytoplasm. The malonyl-CoA produced by ACC1 is funneled into the synthesis and elongation of fatty acids.¹²⁻¹⁴ Acetyl-CoA carboxylase 2 is a 280-kDa protein anchored in the outer mitochondrial membrane and has an additional 136 amino acids in its N-terminus compared with ACC1.¹⁵ Twenty N-terminal hydrophobic amino acids guide ACC2 to the mitochondrial membrane, where the protein co-localizes with carnitine palmitoyl transferase 1. The transferase catalyzes the binding of long-chain fatty acids to carnitine, which transports fatty acids into mitochondria for subsequent β -oxidation.¹⁵⁻¹⁹ The malonyl-CoA produced by ACC2 strongly inhibits carnitine palmitoyl transferase 1.²⁰ Therefore, reduction of acetyl-CoA carboxylase 2 (low malonyl-CoA) activates carnitine palmitoyl transferase 1, thereby increasing the mitochondrial uptake and subsequent β -oxidation of fatty acids and the energy expenditure of cells which may lead to a lean phenotype.²¹

(b) Pyruvate carboxylase (PC) is a 130-kDa protein that forms a homotetramer in mitochondria. PC catalyzes the conversion of pyruvate to oxaloacetate, an intermediate in the biosynthesis of phosphoenolpyruvate and, ultimately, glucose. Evidence from studies in *Drosophila melanogaster*²² and pyruvate carboxylase deficiency caused in humans by an inborn error of metabolism,²³ inborn biotin transporter deficiency,²⁴ or nutritional biotin deficiency²⁵ suggests that biotin deficiency causes an increase in lactate due to accumulation of pyruvate with subsequent reduction of pyruvate to lactate.

(c) Propionyl-CoA carboxylase (PCC) is a heteropolymeric enzyme composed of α and β subunits; biotin is attached to each α subunit, which has a mass of about 80 kDa. PCC

localizes in mitochondria where it catalyzes the covalent binding of bicarbonate to propionyl-CoA, thereby producing β -malonyl-CoA, a key intermediate in the metabolism of odd-chain fatty acids. The two nonidentical subunits that comprise the hexamer of PCC are synthesized in the cytosol with leader peptides that facilitate their transport into the mitochondria,^{26, 27} a feature that they share with other mitochondrial enzymes. Assembly of the subunits occurs after transport and can take place without prior biotinylation.²⁸ In contrast to blood and urinary lactate, metabolites such as 3-hydroxypropionate and 2-methylcitrate, which accumulate in PCC deficiency, are not good indicators of marginal biotin deficiency in humans.²⁹ However, the urinary ratio of propionyl carnitine to methylmalonyl carnitine does increase in the urine in experimental marginal biotin deficiency in humans.³⁰

(d) Methylcrotonoyl-CoA carboxylase (MCC) also is a heteropolymeric enzyme composed of α and β subunits; biotin is attached to each α subunit, which has a mass of about 83 kDa. MCC localizes in mitochondria where it catalyzes the covalent binding of bicarbonate to β -methylcrotonyl-CoA to produce β -methylglutaconyl-CoA, which is a key step in the catabolism of leucine. Loss of biotinylated MCC, e.g., in biotin- or HLCS-deficient persons, leads to the shunting of methylcrotonyl-CoA to alternative pathways and high urinary and plasma levels of 3-hydroxyisovaleric acid and 3-hydroxyisovaleryl carnitine.³¹⁻³⁴

Note that a recent study in biotin-defined outpatients suggests that the inter-individual variation among in free-living subjects is substantial rendering many of the markers inaccurate.³⁵ Although it is possible that the degree of biotin deficiency achieved in that study may not have been as severe as in previously published studies, that study concluded that the expression of genes coding for HLCS, ACC1, ACC2, PC, PCC, and MCC are not good markers for biotin status, the abundance and probably activity of holocarboxylases are excellent markers for biotin status, but the activities of carboxylases were not measured. This study also observed substantial overlap in urinary excretion and large inter-individual variation in the urinary excretion of organic acids is large including that of 3-hydroxyisovaleric acid in subjects on biotin-defined diets. The available markers for biotin status need to be assessed in the light of this study. Concerns regarding inter-individual variability do not apply in studies in cell cultures and animals, where cohorts are homogeneous.^{36, 37}

About 30 mutations have been reported in the *HLCS* gene.³⁸ Mutations occur throughout the entire coding region except exons 6 and 10. The types of mutations are one single amino acid deletion, five single nucleotide insertions/deletions, 22 missense mutations, and two nonsense mutations. The only intronic mutation identified thus far is c.151915G4A (also designated IVS1015G4A), which causes a splice error. There is a good relationship between clinical biotin responsiveness and the residual activity of HLCS. Patients who have mutant HLCS with higher residual activity show a good clinical response to biotin therapy. In addition to these mutations, about 2,600 single nucleotide polymorphisms have been reported for the *HLCS* gene.³⁹ Polymorphisms that result in a change of the amino acid sequence compared with the consensus sequence have been characterized at the kinetics level, using recombinant HLCS in an *in vitro* PCC biotinylation assay.⁴⁰ The biotin affinity of variant Q699R is lower than that of HLCS with the consensus sequence, but the maximal

activity is restored to that of consensus HLCS when assay mixtures are supplemented with biotin. The biotin affinities of HLCS variants V96F and G510R are not significantly different from consensus HLCS, and their maximal activities remain moderately lower than that of consensus HLCS even when assay mixtures are supplemented with biotin. The V96L variant does not alter enzyme kinetics. These findings suggest that individuals with HLCS polymorphisms may benefit from supplemental biotin, yet to different extents depending on the genotype. Consistent with the important roles of HLCS in intermediary metabolism, no living HLCS null individual has ever been reported, suggesting embryonic lethality. Unless diagnosed and treated early, homozygous severe HLCS deficiency is characteristically fatal.⁴¹

Our understanding of the biological functions of HLCS has been expanded substantially beyond its classical roles in carboxylase catalysis. These advances were made possible through developing protocols for making recombinant HLCS of high specific activity,^{42, 43} and the purification of biotinylated human proteins for their subsequent identification by mass spectrometry analysis.⁴⁴ The discoveries resulting from these accomplishments are summarized in the following sections.

Novel Roles of HLCS in chromatin and epigenetics

In 1995, Hymes, Fleischhauer, and Wolf proposed that biotinidase has histone biotinyl transferase activity *in vitro*.⁴⁵ The classical role of biotinidase is to release lysine-bound biotin (biocytin), produced in the breakdown of biotinylated carboxylases.⁴⁶ Zempleni and co-workers soon demonstrated that the binding of biotin to histones is an exceedingly rare, yet natural epigenetic mark,⁴⁷ and that only <0.001% of histones H3 and H4 contain covalently bound biotin.^{48, 49} Studies in fibroblasts from an HLCS-deficient patient suggested a loss of histone biotinylation marks compared with wild-type fibroblasts,⁵⁰ reinforcing previous concerns that HLCS, not biotinidase, catalyzes biotinylation of histones.⁵¹ While there is unambiguous evidence that biotinidase may catalyze the binding of biotin to histones, it is now widely accepted that this reaction is an *in vitro* artifact,^{50, 52} caused by the unphysiologically high concentration of biotinidase in assay reactions. Investigators arrived at this conclusion because knockdown of HLCS, but not biotinidase, produced loss of histone biotinylation marks in *Drosophila melanogaster*; further the loss of HLCS but not of biotinidase produced a phenotype that included shortened life span and low heat stress survival.⁵³

Unambiguous evidence suggests that HLCS enters the nuclear space,^{54, 55} despite lacking a strong nuclear localization signal. There is consensus that HLCS with translational start sites in methionines 1 and 58 can enter the nucleus, whereas HLCS with a translational start site in methionine 7 apparently cannot enter the nucleus. Zempleni and coworkers have speculated that HLCS enters nuclei through physical interactions with other nuclear proteins such as histones H3,⁴³ but experimental confirmation is lacking. Nuclear HLCS is a chromatin protein, judged by chromatin immunoprecipitation in human cell cultures,⁵⁶ immunocytochemistry experiments in human cell cultures,⁵⁷ staining of polytene chromosomes purified from *Drosophila melanogaster*,⁵³ and interactions between HLCS and chromatin proteins (see below).

To date, lysine residues are the exclusive biotinylation sites in histones,^{50, 52, 57} with histones H3 and H4 being the primary targets.⁴⁹ The creation of these marks depends on DNA methylation,⁵⁸ and histone biotinylation marks are also lost in biotin-deficient and HLCS knockdown cells.⁵⁸⁻⁶¹ Despite their rarity, histone biotinylation marks have been consistently found in transcriptionally repressed chromatin, particularly in repeats.⁵⁸⁻⁶³ Loss of HLCS causes a significant loss of K9-methylated histone H3 (H3K9me), which is an abundant and well-established gene repression mark.

Biotin deficiency and HLCS deficiency precipitate severe phenotypes including short life span, low heat stress survival, and loss of histone biotinylation marks in *Drosophila*,⁵³ and de-repression of repeats and chromosomal abnormalities in human cells, mammalian cell cultures, and *Drosophila*.⁵⁸ Recently, a model has been proposed that integrates the above findings into a coherent model to explain the roles of HLCS in gene regulation at the epigenetic level. This model posits that HLCS, which lacks a strong DNA-binding motif, binds to chromatin through interacting with other chromatin proteins, thereby creating a multiprotein gene repression complex (Figure 2). Unambiguous evidence suggests that HLCS interacts with the maintenance DNA methyltransferase DNMT1 (but not with the *de novo* methyltransferases DNMT3a and DNMT3b) and the methyl CpG binding protein MeCP2,⁶⁴ which explains why the binding of HLCS to chromatin and subsequent creation of histone biotinylation marks depends on DNA methylation marks.⁵⁸ HLCS also interacts physically with the euchromatic histone-lysine methyltransferase EHMT1.⁶⁵ HLCS catalyzes the biotinylation of K161 and other lysine residues in EHMT1, thereby strengthening the interaction between the two proteins *in vitro*⁶⁵; the relevance of this observation *in vivo* is not yet clear. EHMT1 is among the methyltransferases responsible for creation of H3K9me gene repression marks, which is mediated by the binding of heterochromatin protein 1 (HP1) to H3K9me marks.⁶⁶ The binding of EHMT1 to HLCS explains the loss of H3K9me marks and the de-repression of repeats in HLCS- and biotin-deficient cells.^{58, 60} The interactions between HLCS and the methyltransferases DNMT1 and EHMT1 also suggests the possibility of epigenetic synergies between biotin and methyl donors such as folate, S-adenosyl methionine, and perhaps choline. Evidence suggests that HLCS, biotin, and methyl donors synergize in the repression of loci that are predominantly regulated by epigenetic mechanisms, e.g., long-terminal repeats; however, these synergies cannot be reproduced in genes coding for cytokines, which are regulated by a multitude of transcriptional regulators.⁶⁷ Finally, the same algorithm that predicted physical interactions between HLCS and EHMT1 also predicted interactions between HLCS and the nuclear co-repressor N-CoR2.⁶⁵ N-CoR recruits histone deacetylases to chromatin, thereby contributing to gene repression through removing histone acetylation marks.⁶⁶ Zempleni and Liu currently test whether HLCS interacts physically with histone deacetylases and N-CoR. The proposed model is consistent with the ability of HLCS to catalyze biotinylation of histones,⁴³ but acceptance of the proposed model implies that histone biotinylation marks are a mere side effect of HLCS being in close physical proximity to histones, mediated by interactions with a variety of chromatin proteins. According to this model, biotinylation marks play no important role in gene repression, despite contributing toward chromatin condensation,^{68, 69} but instead mark HLCS docking sites in chromatin. This notion is

consistent with previous reports suggesting that biotinylation marks are barely detectable in histone H2A,^{47, 70} and that any biotinylation of histone H2A might be nonenzymatic.⁷¹

Novel biotinylated proteins

A recent mass spectrometry screen revealed the identities of 108 biotinylated proteins in human embryonic kidney HEK293 cells.⁴⁴ Heat shock proteins (HSP), enzymes with roles in glycolysis and protein biosynthesis, and proteins with functions in the cytoskeleton (tubulin and actin) were greatly overrepresented among biotinylated proteins. The characterization of some of these proteins, including the role of biotinylation in protein function, has been initiated and is summarized in the following sections. Some of these biotinylation events may have no physiological relevance, considering that HLCS is a rather promiscuous enzyme regarding its biotinylation targets. For example, the microbial ortholog of HLCS, BirA, catalyzes the biotinylation of eukaryotic proteins including histones and the PCC fragment, p67.⁷² Note, however, that HLCS does not biotinylate every available lysine residue suggesting the existence of a certain degree of specificity. For example, when a variant of p67, Syn67, was incubated with HLCS, no biotinylation of lysines in Syn67 was apparent.⁷³

Biotinylation of heat-shock proteins—Ongoing studies focus on the biotinylation of two HSPs, i.e., HSP72 and HSP60. The stress-inducible HSP72 localizes in cytoplasm, nucleus and extracellular space and is essential for unfolded protein response and immune function.^{74, 75} The level of extracellular HSP72 increases in response to bacterial infection in rats,⁷⁶ treatment with lipopolysaccharides in THP-1 human monocytic cells,⁷⁷ and inhibition of phospholipase C in A431 human squamous carcinoma cells.⁷⁸ Extracellular HSP72 promotes the secretion of proinflammatory cytokines.^{76, 79, 80} Importantly, previous studies conducted chemical, not site-specific biotinylation of HSP72 to create HSP72bio.⁸¹ When HEK293 cells were incubated with HSP72bio, the secretion of the chemokine RANTES increased significantly compared with cells treated with non-biotinylated HSP72.

Our mass spectrometry studies revealed that K112, K128, K348, K361, and K415 are targets for biotinylation in HSP72.⁴⁴ HLCS interacts physically with HSP72, judged by co-immunoprecipitation in combination with western blot analysis and mass spectrometry. Recombinant HSP72 was biotinylated successfully using recombinant HLCS. Consistent with previous studies using chemically biotinylated HSP72, enzymatically biotinylated HSP72 also increases the secretion of RANTES by HEK293. This effect appears to not depend on the biotinylation of a particular lysine residue, raising the possibility that posttranslational modifications other than biotinylation may also affect the activity of eHSP72.

A second study of HSP focused on HSP60, because of its pivotal role in ameliorating oxidative stress.⁸² HSP is unique in that the protein is dually modified by methionine sulfoxidation and lysine biotinylation (underlined) in the C-terminal KEEKDPGMGAMGGMGGMGGMF motif (Li, Y. et al., submitted). The levels of reactive oxygen species are about ten times higher in biotin-depleted HEK293 cells compared with biotin-sufficient controls, and the biotin-depleted cells arrest in G0/G1 and S

phases of the cell cycle. A causal relationship between biotinylation and sulfoxidation of HSP60 remains to be established; however, initial observations offer intriguing insights into possible new roles for biotin and HLCS in redox biology.

Biotinylation of enolase-1 and MBP-1—The human ENO1 gene codes for enolase-1, the c-myc promoter binding protein MBP-1, and tau-crystallin.⁸³ Enolase-1 (ENO1) catalyzes a key reaction in glycolysis, i.e., the conversion of 2-phosphoglycerate to phosphoenolpyruvate.¹³ MBP-1 is a tumor suppressor transcription factor domain that binds and represses the c-myc and cyclooxygenase 2 (COX2) promoters, thereby reducing the expression of the *c-myc* and COX2 oncogenes.^{84, 85} Repression of these oncogenes has important implications for cancer risk. For example, high c-myc activity associates with poor prognosis in breast cancer,⁸⁶ and high COX2 levels promote the growth and metastasis of gastric cancer cells.⁸⁵ The amino acid sequence of MBP-1 is identical to that of ENO1, but it lacks the 93 N-terminal amino acids (GenBank entries AAH50642.1 for ENO1 and NP_001188412 for MBP-1). Tau-Crystallin is a structural protein found in eye lenses.⁸⁷

Mass spectrometry analyses suggest that K10 and K12 are biotinylated in MBP-1.⁸⁸ Biotinylation of MBP-1 is catalyzed by HLCS, judged by *in vitro* assays using recombinant HLCS and MBP-1.⁸⁸ The abundance of biotinylated MBP-1 depends on the concentration of biotin in cell culture media in HEK293 cells.⁸⁸ Importantly, the activity of a c-myc reporter gene is inversely linked with biotin in culture media in HEK293 cells. Likewise the abundance of mRNA coding for c-myc and COX2 is inversely linked with biotin in culture media in HEK293 cells. Consistent with these observations, loss or mutation of HLCS is reported in three independent human cancer patent databases strongly supporting its association with tumorigenesis.⁸⁹⁻⁹¹

Conclusion

There is no evidence that HLCS has roles in gene regulation, chromatin biology, and intermediary metabolism that go far beyond its classical role in mediating carboxylase-depending reactions in fatty acid metabolism, gluconeogenesis, and leucine metabolism. For some of these novel roles, there already is a strong body of evidence (epigenetics, HSPs, MBP-1) whereas for others novel roles are just emerging. Our laboratory is currently in the process of creating a conditional HLCS knockout mouse, which will be a valuable tool in facilitating studies of novel roles of HLCS.

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Figure 1. Biotin-dependent carboxylases

Five biotin-dependent carboxylases participate in the metabolism of fatty acids, glucose, and leucine in cytoplasm and mitochondria. Abbreviations: ACC1 and ACC2, acetyl-CoA carboxylases 1 and 2; MCC, methylcrotonyl-CoA carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase.

Figure 2. HLCS-containing multiprotein gene repression complex

Note that the participation of HDAC and N-CoR has not yet been experimentally confirmed.

Abbreviations: ac, acetyl; bio, biotin; DNMT1, DNA methyl transferase 1; EHMT1, euchromatic histone methyl transferase 1; H3K9me, K9-methylated histone H3; HDACs, histone deacetylases; HLCS, holocarboxylase synthetase; HP1, heterochromatin protein 1; MBD, methyl binding domain proteins; me, methyl; MeCP2, methyl CpG binding protein; N-CoR, nuclear co-repressor; S-Ad-Met, S-adenosyl methionine.