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Molecular genetics of biotin metabolism: old vitamin, new science☆

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Abstract

Biotin is a water-soluble vitamin that participates as a cofactor in gluconeogenesis, fatty acid synthesis and branched chain amino acid catabolism. It functions as the carboxyl carrier for biotin-dependent carboxylases. Its covalent attachment to carboxylases is catalyzed by holocarboxylase synthetase. Our interest in biotin has been through the genetic disease, "biotin-responsive multiple carboxylase deficiency," caused by deficient activity of holocarboxylase synthetase. As part of these studies, we made the unexpected findings that the enzyme also targets to the nucleus and that it catalyzes the attachment of biotin to histones. We found that patients with holocarboxylase synthetase deficiency have a much reduced level of biotinylated histones, yet the importance of this process is unknown. The dual nature of biotin, as the carboxyl-carrier cofactor of carboxylases and as a ligand of unknown function attached to histones, is an enigma that suggests a much more involved role for biotin than anticipated. It may change our outlook on the optimal nutritional intake of biotin and its importance in biological processes such as development, cellular homeostasis and regulation.

Keywords: Biotin; Holocarboxylase synthetase; Carboxylases; Histones; Nucleus; Multiple carboxylase deficiency

1. The biotin cycle and the biotin-dependent carboxylases

Biotin has been recognized as an essential nutrient since the early part of the last century. We now appreciate that our biotin requirement is fulfilled in part through diet, through endogenous reutilization of biotin and perhaps through capture of biotin generated in the intestinal flora [1]. The utilization of biotin for covalent attachment to carboxylases and its reutilization through the release of carboxylase biotin after proteolytic degradation constitutes the "biotin cycle." Biotin deficiency is associated with neurological manifestations, skin rash, hair loss and metabolic disturbances that are thought to relate to the various carboxylase deficiencies (metabolic ketoacidosis with lactic acidosis). It has also been suggested that biotin deficiency is associated with protein malnutrition [2,3], and that marginal biotin deficiency in pregnant women may be teratogenic [4]. While these data highlight the importance of biotin as an essential nutrient, the role of biotin in cells remains incompletely understood.

Biotin acts as a carboxyl carrier in carboxylation reactions [1]. There are four biotin-dependent carboxylases in mammals: those of propionyl-CoA (PCC), β-methylcrotonyl-CoA (MCC), pyruvate (PC) and acetyl-CoA carboxylases (isoforms ACC-1 and ACC-2). All but ACC-2 are mitochondrial enzymes. The biotin moiety is covalently bound to the ε amino group of a Lys residue in each of these carboxylases in a domain 60–80 amino acids long. The domain is structurally similar among carboxylases from bacteria to mammals. At the center is a short peptide, (A/V)MKM, which is the near universal biotin acceptor sequence. HCS catalyses the transfer of biotin to all of the apocarboxylase substrates.

Our knowledge of the reaction mechanism of HCS comes from studies of the orthologous *Escherichia coli* protein, BirA. It functions both as the biotin transfer enzyme and as the repressor of the biotin biosynthetic operon [5]. Biotin is transferred, via a two step reaction involving a biotin-5′ -AMP intermediate, to the biotin carboxyl carrier protein (BCCP) of ACC with consequent release of AMP.

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Xu and Beckett [6] showed that binding of biotin-AMP to BirA produces a significant conformational shift (allosteric effect) that stabilizes the BirA-biotin-AMP complex. This prevents unproductive synthesis and release of biotin-AMP when BCCP is fully saturated with biotin. The BirA-biotin-AMP complex also shifts to a dimer form which constitutes the active repressor of the biotin operon [7–9].

The cloning of the cDNA for HCS made it possible to compare its sequence structure with that of BirA [10,11]. While HCS and BirA share the biotin ligase function, their structural relatedness is limited to ~130 amino acids (60% identity) comprising the biotin transfer domain, despite lengths of 726 amino acids for HCS and 325 for BirA. The N-terminal half of BirA is concerned primarily with its repressor function and contains the domain for DNA binding. The function of the N-terminal half of HCS remains unknown. For both proteins, the N-terminal half can be eliminated without disrupting its biotin ligase function [6,12]. The structure of the *HLCS* gene (ENSG00000159267) has been determined. It is located on chromosome 21q22.1 and consists of 14 exons and 13 introns in a span of 240 kb [13].

Among the carboxylases, genetic diseases have been described for all four enzymes individually [1]. In MCD, all carboxylase activities are simultaneously deficient owing to universally defective biotinylation. There are two genetically distinct causes: mutations in HCS or in biotinidase (BTD). In HCS deficiency, the defect is in biotin transfer to apocarboxylases. In BTD deficiency, the defect lies in blocking reentry of biotin into the biotin cycle due to defective release of biotin from biocytin (biotinyllysine), the product of the proteolysis of carboxylases. HCS deficiency may be life threatening during infancy. Biotinidase deficiency tends to be of later onset and has milder symptoms [1]. Both diseases can be treated with pharmacological supplementation with biotin, although those with BTD deficiency retain hearing and visual deficits that can be avoided through presymptomatic screening. Both enzymes are found in all cells, and BTD is also a significant plasma glycoprotein. Burri et al. [14,15] showed that most patients with defective HCS had a decreased affinity for biotin, and, following the cDNA cloning, we and others showed that most patient mutations occur in the biotin binding region of HCS, consistent with Burri's expectations [13,16].

2. The nucleus, histones and biotin

Early studies revealed that a large proportion of radioactive biotin injected into chicks and rats localized to the nuclear fraction of cells [17]. Some studies have reported biotin in nuclei of tumor material and normal tissues [18–20]. The main source of nuclear biotin may be through covalent attachment to histones. Stanley et al. [21] showed that all five histone classes extracted from human lymphocytes contain biotin that was detected by Western blot using streptavidin or anti-biotin. This experiment derives from the earlier discovery by Hymes et al. [22] that BTD will exchange biotin between biocytin and histones in vitro. The reaction was demonstrated with serum samples and purified BTD and was deficient when sera of patients with BTD deficiency were used. The reverse reaction, removal of biotin from histones, was also demonstrated using human plasma or lymphocyte extracts and was also deficient in patient samples [23]. The reaction did not work with free biotin as substrate.

Independently of these studies, we generated polyclonal antibodies to HCS to permit subcellular localization of the enzyme. At issue was whether biotinylation of mitochondrial carboxylase occurs within the mitochondria or in transit in the cytosol. Three antibodies were produced, two against peptides corresponding to sequences at the mature N- and C-terminus of HCS (residues, 58–77 and 707–726, respectively) and a third to near full-length HCS (residues, 58–726) that was expressed in *E. coli*. While the antibodies were used to examine the cytoplasmic distribution of HCS, they instead showed it primarily in the nucleus of HeLa, Hep2 and fibroblasts with only a minor component in the cytoplasm [24]. This was completely unexpected, but the same result was obtained by transfecting recombinant HCS containing a C-terminal tag. HCS localized to the core nuclear lamina in cells progressively extracted through increasing salt and DNase I treatment. This parallels immunofluorescent detection of lamin B, except that the HCS distribution was discontinuous. DNase I did not significantly alter its distribution, suggesting that HCS is not tightly associated with chromatin. HCS was also excluded from condensed chromosomes and was found to be dispersed as particulate ring-like structures in all phases of mitosis. Given this distribution, mitotic Hep2 and HeLa cells were examined for possible colocalization of HCS with lamin B. This was done in three dimensions by the analysis of optical stacks that showed >98% colocalization of HCS with lamin B. The in situ results were confirmed by Western blot of fractionated HeLa and Hep2 cells. All three antibodies recognized two protein species of 68 and 66 kDa primarily in the chromatin and nuclear matrix fractions. These results suggest that particulate structures containing HCS derive from the disassembly of the nuclear envelope and indicate that their macromolecular nature is retained, rather than disaggregated, during mitosis.

Assay of biotin transfer to p67, a peptide comprised of the C-terminal 67 amino acids of the PCC α subunit, showed that HCS from the nuclear matrix and insoluble fractions retain biotinylating activity. Given the report of biotinylated histones [21], we assayed purified recombinant enzyme and showed that it could transfer ^{14}C-biotin to all histone classes (H1, H2A, H2B, H3 and H4) in the presence of ATP [24]. Significantly, the substrate was free biotin. This result led us to examine the state of histone biotinylation in MCD cells deficient in HCS activity. Histones were extracted from control and patient fibroblasts and evaluated by avidin and anti-biotin Western blot. In control cells, all five histone

classes were detected by Coomassie stain and all contained biotin. In contrast, mutant cells showed similar histone levels but were profoundly diminished in biotinylation across all five classes. These results provide convincing evidence that HCS is responsible for histone biotinylation. We suggest that BTD, which requires biocytin as substrate, does not attach biotin to histones in vivo. Rather, it may well be responsible for removal of biotin from histones as it does for carboxylase biotin.

Unexpectedly, we also observed that nuclear HCS contains attached biotin—unexpected because HCS does not contain a consensus biotin attachment site (i.e., (A/V)KMK). Nevertheless, anti-biotin capture of proteins from HeLa cell nuclei followed by Western blot with streptavidin revealed a series of biotin-containing proteins, including bands with the pattern of histones [24]. One of the bands, at 68 kDa, was also detected with anti-HCS. The reciprocal experiment, captured with anti-HCS and Western blot with antibiotin, also revealed a 68-kDa band. Blotting with anti-HCS confirmed the presence of both the 68- and 66-kDa HCS species. We also showed that the 66-kDa species from permeabilized interphase or mitotic cells could be solubilized with the nonionic detergent NP-40 while the 68-kDa species remained in the pellet. This reinforces the view that the 68 kDa species is associated with other proteins or cell structures.

These results suggest additional roles for biotin and HCS beyond attachment of the biotin cofactor to carboxylases. There have been long standing suggestions of a role for biotin in genetic regulation. Biotin has been reported to stimulate the synthesis of hepatic glucokinase [25,26] and to repress phosphoenolpyruvate carboxykinase activity [27] in rat liver. It was reported to stimulate an increase in the mRNA for 6-phosphofructokinase following biotin administration to biotin-deficient rats [28,29]. It was also shown to increase the level of the asialoglycoprotein receptor in hepatoma cells using a mechanism that appears to function through cGMP and guanylate cyclase [30–32]. More recently, Stanley et al. and Crisp et al. [21,33] suggested that cell proliferation is linked to biotin content.

Significantly, biotin appears to regulate the expression of HCS and some carboxylases. Solorzano-Vargas et al. [34] reported that in biotin-starved human hepatoma cells, the level of the mRNAs for HCS, ACC-1 and the α subunit of PCC were all reduced and were restored to initial levels on resupplementation with biotin. They showed that the effect was RNA synthesis dependent and that cells from a patient with MCD required 100× the biotin level used for control cells to restore the mRNAs to starting levels. They showed that cGMP can bypass the biotin requirement, in control or MCD cells, and that inhibitors of soluble guanylate cyclase or of cGMP-dependent protein kinase inhibited the cGMP effect. Given the involvement of HCS in the sequence of events, they suggested that biotin-AMP, the intermediate product of the HCS reaction, is a component of the stimulatory process. Independent studies on biotin-deficient rats demonstrated a similar effect on HCS mRNA and protein and on PCC and PC protein levels [35]. Subsequent studies in a rat model revealed that this mechanism of regulation was restricted to tissues such as liver and kidney, while in the brain, the biotin cycle remained constitutively expressed. Leon Del Rio proposed that this pattern of regulation is aimed at restricting biotin utilization in peripheral tissues while sparing the brain during periods of biotin deprivation. These studies suggest a role for biotin that goes beyond maintenance of functional carboxylase activities and suggest further that biotin-AMP may be an intermediate in this process.

In summary, these experiments reveal a dual role for HCS: its traditional role in the biotinylation of carboxylases and a novel role in the attachment of biotin to histones. Our studies suggest a complex pathway of nuclear localization, retention and organization and deficient histone biotinylation in MCD cells. The current studies of the role of biotin in cells may change our outlook on its optimal nutritional intake and the meaning behind the symptoms of individuals with biotin deficiency, genetic or acquired. A benefit of these studies will be to increase our understanding of the disease in MCD and the long-term impact of treatment with pharmacological doses of biotin.

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