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Effects of Single Nucleotide Polymorphisms in the Human *Holocarboxylase Synthetase* Gene on Catalytic Activity

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EFFECTS OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE HUMAN HOLOCARBOXYLASE SYNTHETASE GENE ON CATALYTIC ACTIVITY

By

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Holocarboxylase synthetase (HCS) catalyzes the covalent binding of biotin to carboxylases and histones in eukaryotic cells. Biotinylated carboxylases play essential roles in the metabolism of fatty acids, amino acids, and glucose; biotinylated histones play essential roles in gene regulation and genome stability. HCS null individuals are not viable whereas HCS deficiency is linked to developmental delays and phenotypes such as short life span and low stress resistance. Greater than 2,500 single nucleotide polymorphisms (SNPs) have been reported for HCS, but the biological importance of these polymorphisms is unknown. We hypothesized that some of these SNPs impair catalytic activity and that this effect can be overcome by dietary intervention with biotin. Here, we analyzed the enzyme kinetics of five recombinant HCS variants using a propionyl-CoA carboxylase surrogate ("p67") as substrate for biotinylation. V_{max} of variants L216R, V96F and G510R were 6%, 78% and 73%, respectively, of the V_{max} in wild-type HCS. The K_m values of the variants V96F and G510R were not significantly different from wild-type HCS. The activity of L216R was too low to allow for

meaningful analysis of K_m. In contrast, the affinity of variant Q699R for biotin was significantly lower than that of wild type HCS (K_m: 1.57 times that of wild type) and its V_{max} could be restored to that of wild-type HCS by biotin supplementation. This is the first biochemical characterization of catalytic activities of HCS variants. Also, this is the first report to show that HCS activity can be restored to normal by biotin supplementation.

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Thank you for supporting my research and my education these past two years. I recognize your willingness to give of your time and energies as I had need, and I do not take your sacrifice lightly. I count it an honor to have been trained by a superior researcher and professor.

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Thank you for the countless hours you have spent training me in laboratory practice and imparting sound research philosophy. Apart from your efforts, a graduate education of the caliber that I have received would not have been possible.

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INTRODUCTION

Biotin is a water-soluble vitamin involved in many metabolic pathways in mammals [Camporeale and Zempleni 2006]. It plays important roles in the metabolism of glucose, amino acids and fatty acids due to its role as a coenzyme in the metabolism of acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2), 3-methylcrotonyl-CoA carboxylase (MCC), pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC) [Camporeale and Zempleni 2006; Chew, Sarath et al. 2007]. Biotin is also attached covalently to at least 11 distinct lysine residues in histones H2A, H3 and H4 [Camporeale, Shubert et al. 2004; Kobza, Camporeale et al. 2005; Chew, Camporeale et al. 2006]. Biotinylation of histones participates in the cross-talk among various histone modifications and in gene regulation [Camporeale, Giordano et al. 2006; Chew, Raza et al. 2006; Camporeale, Zempleni et al. 2007; Gralla, Camporeale et al. 2008].

Holocarboxylase synthetase (HCS, EC 6.3.4.10) is an enzyme that catalyzes biotinylation of the five human biotin-dependent carboxylases mentioned above (ACC1, ACC2, MCC, PC, and PCC). The following four domains have been identified and characterized in human HCS: N-terminal domain, central domain, linker domain, and C-terminal domain [Hassan, Moriyama et al. 2009]. Both N- and C-termini of HCS participate in substrate recognition [Hassan, Moriyama et al. 2009]. The central domain in HCS contains binding sites for both ATP and biotin [Leon-Del-Rio and Gravel 1994; 1999; Kothapalli, Sarath et al. 2005]. The linker domain might play a role in conferring the flexibility needed for HCS to interact with a diverse group of substrates for biotinylation [Hassan, Moriyama et al. 2009].

A series of mutations have been identified in the HCS gene; these mutations decrease HCS activity substantially and cause features such as exfoliative dermatitis, hypoglycemia, ketoacidosis, and reduced histone biotinylation. While the prevalence of HCS mutations is low in the general population, early diagnosis is critical for a good prognosis. Mutations that decrease the affinity of HCS for biotin are particularly responsive to treatment with pharmacological doses of biotin. In addition, greater than 2,500 SNPs have been reported for HCS in public databases. The biological importance of these polymorphisms is unknown.

This thesis has the following two major goals. (1) *To identify HCS SNPs that affect the catalytic activity of HCS.* This aim tests the hypothesis that some, but not all, SNPs in human HCS decrease enzyme activity. Previous studies revealed that domains in the N-terminus, ATP-/biotin-binding site (central domain), and C-terminus are important for HCS activity [Hassan, Moriyama et al. 2009]. From each of these three domains, one to two known SNPs were selected that were likely to affect catalytic activity. Candidate SNPs were those suspected to cause HCS conformational changes, e.g., substitution of hydrophobic for basic amino acids. (2) *To determine if higher concentration of biotin could restore substrate biotinylation by HCS variants to the levels of wild-type HCS.* This aim tests the hypothesis that supplementation with biotin increases the catalytic activity of HCS variants to levels seen with wild-type HCS. These supplementation studies lay the groundwork for future dietary intervention studies.

LITERATURE REVIEW

I. Biotin

Biotin, also known as vitamin B7 or H, is an essential water-soluble vitamin involved in many metabolic pathways in mammals [Zempleni 2005]. Chemically, biotin is a monocarboxylic acid consisting of a ureido group, thiophane heterocyclic ring, and a valeric acid side chain [Zempleni 2001; Zempleni 2005; Hassan and Zempleni 2006] (**Fig. 1**).

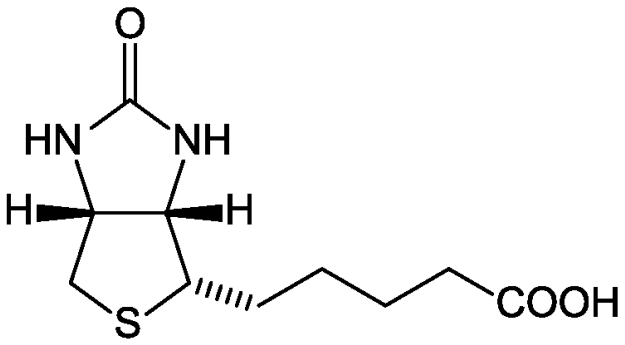


Figure 1. Biotin structure

Biotin is a coenzyme in carboxyl transfer reactions from microbes to animals and plants. Biotin was first discovered by Kögl and Tönnis in 1934 while studying the growth requirements of yeast in synthetic media [Kogl and Tonnis 1932]. Mammals are auxotrophic for biotin and obtain it through dietary sources and from intestinal bacteria that can produce biotin *de novo* [Wakil, Stoops et al. 1983], or endogenously recover it

from pre-existing carboxylases. Biotin can be found in high concentration in egg yolk, cow milk, liver and some vegetables [Said 2009]. For absorption of dietary biotin, the protein-bound biotin in foods has to be converted to free biotin by the action of gastrointestinal proteases, peptidases, and biotinidase [Zempleni 2005; Said 2009]. The adequate intake (AI) of biotin for adults is 30 µg /day.

The following four proteins play major roles in the homeostasis of biotin in humans (**Fig. 2**): the sodium-dependent multi-vitamin transporter (SMVT), monocarboxylate transporter 1 (MCT1), biotinidase (BTD) and holocarboxylase synthetase (HCS**).** In most mammalian tissues the primary transport protein of biotin is SMVT, which also transports pantothenic acid and lipoate [Wang, Huang et al. 1999; Said 2009]. MCT1 has been reported to participate in the transport of biotin, but its function is restricted to lymphoid cells [Zempleni and Mock 1999; Daberkow, White et al. 2003]. In mammals, biotin reutilization occurs through the action of BTD, a specific amidolyase which hydrolyzes biotin from biotinyl-lysine (biocytin) or short peptides containing biotin which are the products of normal proteolytic digestion of holocarboxylases [Wolf, Grier et al. 1985]. BTD is also required for the release of dietary protein-bound biotin.

Within the cell, the most extensively characterised and understood function for biotin is to serve as a cofactor for biotin-dependent carboxylases (propionyl-CoA carboxylase (PCC), cytoplasmatic acetyl-CoA carboxylase 1 (ACC1), mitochondrial acetyl-CoA carboxylase 2 (ACC2), pyruvate carboxylase (PC) and 3-methylcrotonyl-CoA carboxylase (MCC)) where it is required for the transfer of carbon dioxide from bicarbonate to organic acid metabolites and plays important roles in the metabolism

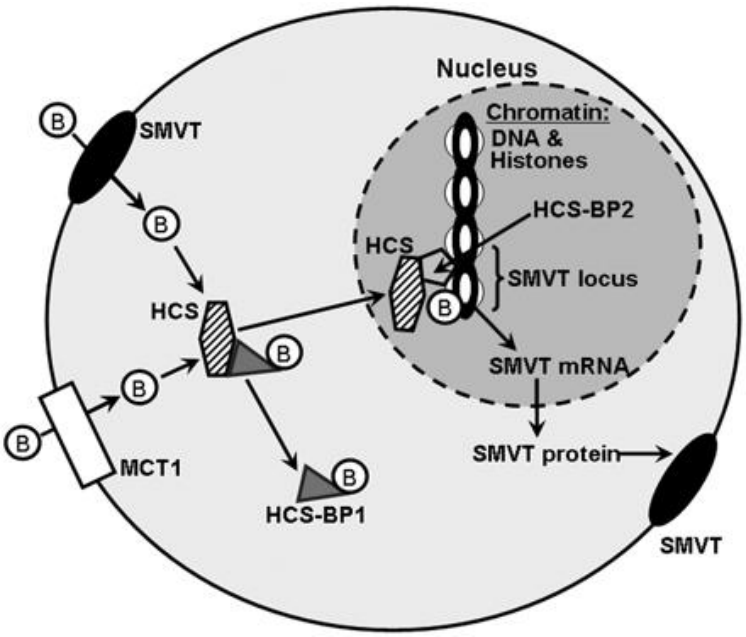


Figure 2. Biotin transport and metabolism. B: biotin, SMVT: sodium multivitamin transporter, MCT1: monocarboxylate transporter 1, HCS: holocarboxylase synthetase, PCC: propionyl-CoA carboxylase, ACC1: cytoplasmatic acetyl-CoA carboxylase α, ACC2: mitochondrial acetyl-CoA carboxylase β, PC: pyruvate carboxylase, MCC: and 3-methylcrotonyl-CoA carboxylase [Figure source: Zempleni, Gralla et al. 2009].

of glucose, amino acids and fatty acids [Wood and Barden 1977; Samols, Thornton et al. 1988; Knowles 1989; Attwood and Wallace 2002]. Biotin is covalently attached to this family of enzymes by biotin protein ligase (BPL). Biotin-dependent carboxylases have half-lives of 1–8 days [Majerus and Kilburn 1969; Nakanishi and Numa 1970; Weinberg and Utter 1979; Weinberg and Utter 1980; Freytag and Utter 1983]. Both ACC1 and ACC2 catalyze the incorporation of bicarbonate into malonyl-CoA, a key regulatory step in fatty acid synthesis (ACC1) and mitochondrial fatty acid transport (ACC2). MCC is

involved in the catabolism of the branched-chain amino acid leucine. PC catalyzes the carboxylation of pyruvate to produce oxaloacetate in gluconeogenesis. PCC is involved in the conversion of propionyl-CoA to methylmalonyl-CoA in the metabolism of odd-chain fatty acids.

II. Holocarboxylase Synthetase (HCS or HLCS)

The Role of HCS in Humans

HCS is a biotin protein ligase (BPL): an enzyme that catalyzes biotin attachment onto the biotin-dependent enzymes - carboxylases and histones – in mammalian cells [Leon-Del-Rio and Gravel 1994; Narang, Dumas et al. 2004]. HCS catalyzes the biotinylation of the five mammalian biotin-dependent carboxylases mentioned above (ACC1, ACC2, MCC, PC, and PCC). The covalent bond between the lysine residue of the carboxylases and biotin occurs in a two-step ATP-dependent reaction [Dakshinamurti and Chauhan 1994]. In the first step, biotinyl 5'-AMP (B-AMP) is generated; in the second step, biotin is transferred from B-AMP to the lysine residue in the apocarboxylases [Narang, Dumas et al. 2004] (**Fig. 3**).

In addition, in response to changes in biotin availability, HCS has been demonstrated to function in regulating transcription of the gene that encodes HCS itself as well as those that code for propionyl-CoA carboxylase, pyruvate carboxylase, acetyl-CoA carboxylase 1, and the sodium-dependent multivitamin transporter [Dakshinamurti and Cheah-Tan 1968; Chauhan and Dakshinamurti 1991]. This transcriptional regulatory process is associated with the soluble guanylyl cyclase signal transduction pathway

[Solorzano-Vargas, Pacheco-Alvarez et al. 2002]. It has also been suggested that HCS exerts its transcriptional regulatory role by catalyzing biotin linkage to histones [Zempleni 2005] (**Fig. 4**).

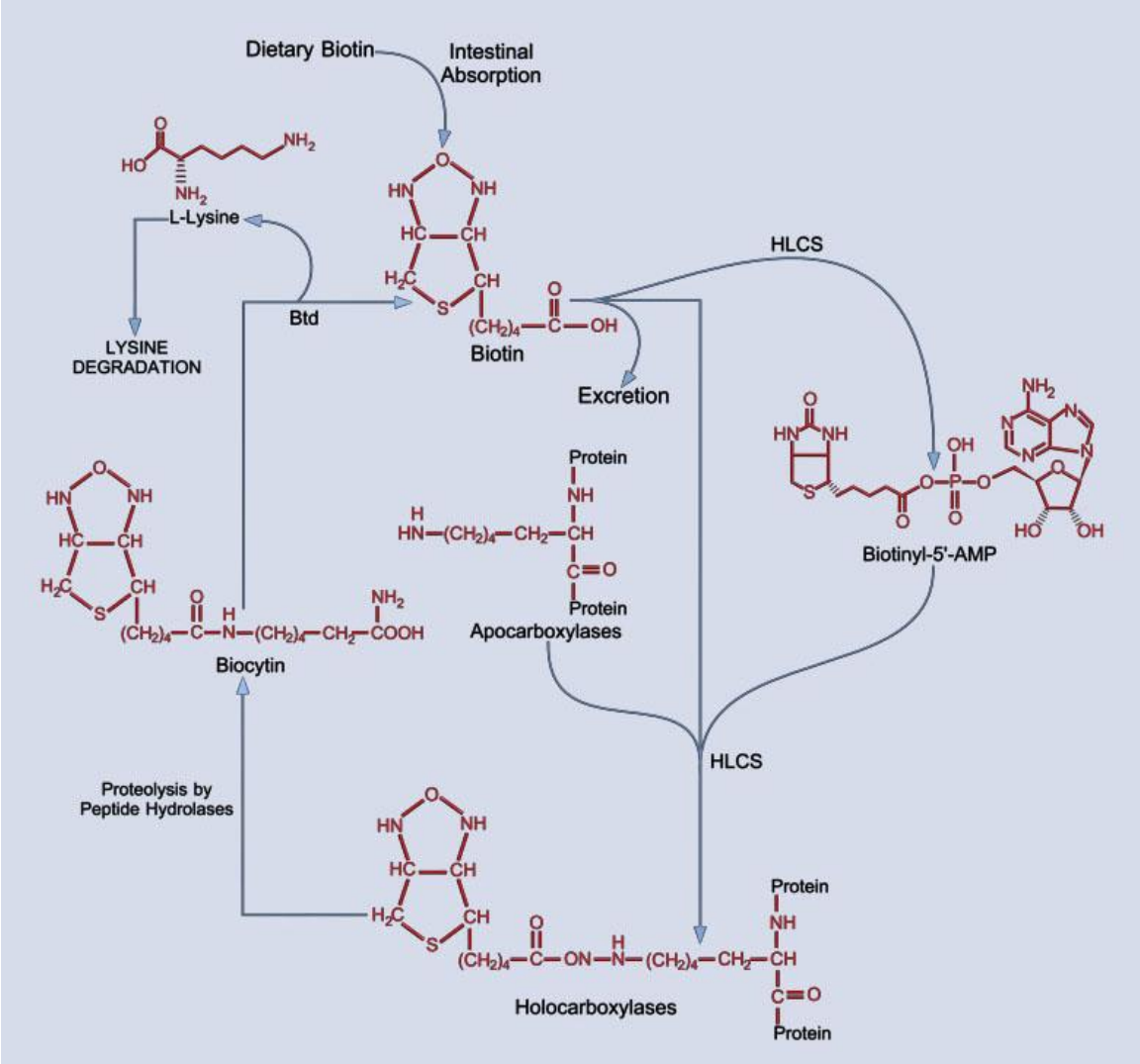


Figure 3. Biotin metabolism in *Homo sapiens*. Btd: biotinidase; HLCS: holocarboxylase synthetase; apocarboxylase: unbiotinylated carboxylase; holocarboxylase: biotinylated carboxylase (Figure source: QIAGEN; Valencia, CA)

The carboxylases mentioned above are essential for cellular biosynthesis.

Therefore, defects in HCS, which reduce the biotin-dependent enzyme activity, affect several important metabolic processes, including fatty acid synthesis, gluconeogenesis, and amino acid catabolism.

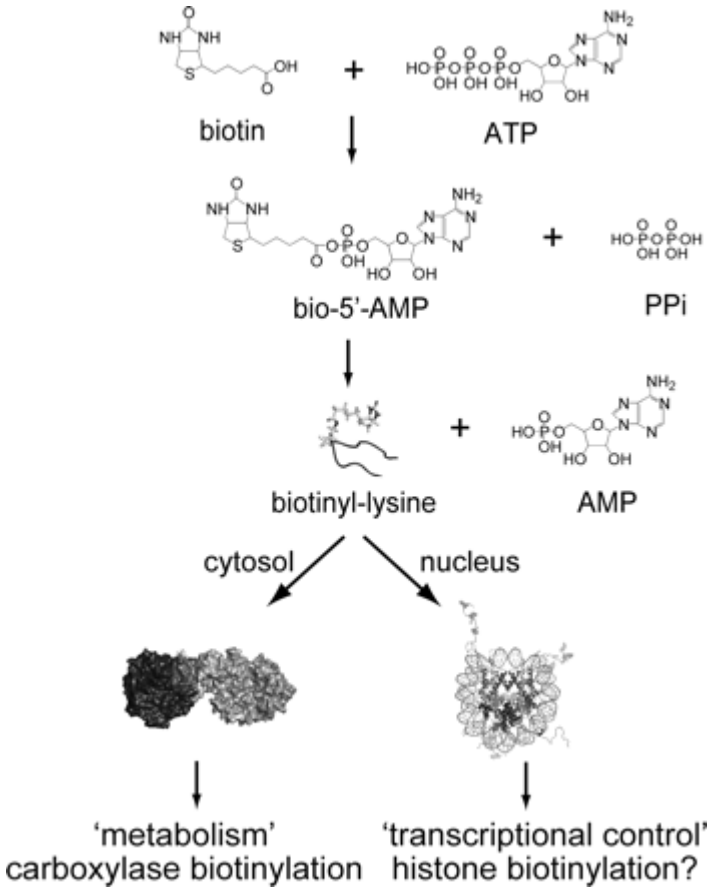


Figure 4. HCS function in metabolism and transcription regulation. The enzyme utilizes substrates biotin and ATP to catalyze synthesis of bio- 5′-AMP. The HCS ·intermediate complex interacts with carboxylases to transfer biotin or functions in transcription, perhaps through histone biotinylation [Figure Source: Ingaramo and Beckett 2009].

HCS Structure and Domains

Holocarboxylase synthetase (HCS or HCS, EC 6.34.10) is encoded by an 14-exon gene, *HCS,* located on chromosome 21q22.1 [Suzuki, Aoki et al. 1994]. The open reading frame of full-length HCS encodes 726 amino acids (2181bp) [Suzuki, Aoki et al. 1994]. The existence of at least three splicing variants (76, 82, and 86 kDa) has been confirmed [Hiratsuka, Sakamoto et al. 1998]. HCS has been detected in cytoplasm, mitochondria, cell nuclei, and the nuclear lamina [Hiratsuka, Sakamoto et al. 1998; Narang, Dumas et al. 2004].

The following four domains have been identified and characterized in human HCS: N-terminal domain, central domain, linker domain, and C-terminal domain [Hassan, Moriyama et al. 2009] (**Fig. 5**). Both N- and C-termini of HCS participate in substrate recognition [Hassan, Moriyama et al. 2009]. The central domain in HCS contains binding sites for both ATP and biotin [Leon-Del-Rio and Gravel 1994; Chapman-Smith and Cronan 1999; Kothapalli, Sarath et al. 2005]. The linker domain might play a role in conferring the flexibility needed for HCS to interact with a diverse group of substrates for biotinylation [Hassan, Moriyama et al. 2009].

The protein HCS has a homologous region (aa 448-701) to BirA, the biotin apo-carboxyl carrier protein ligase of Escherichia coli [Suzuki, Aoki et al. 1994]. BirA ligase participates in the biotinylation of biotinoyl carboxyl carrier protein (BCCP) and its amino acid sequence is 21% identical with HCS [Chauhan and Dakshinamurti 1986; Bankson, Martin et al. 1987; Hymes and Wolf 1999; Hymes, Stanley et al. 2001].

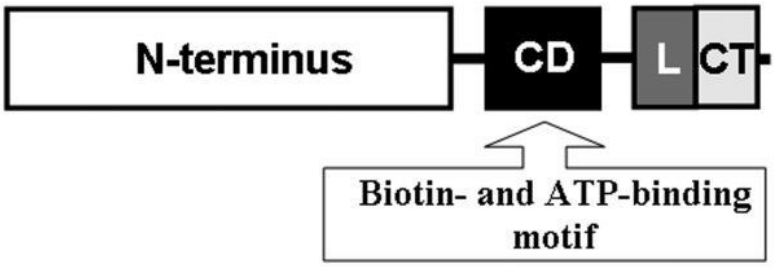


Figure 5. Four domains in human HCS: N-terminal domain (M_1-F_{446}); central domain "CD" (F_{471}-S_{575}) containing biotin transfer and ATP-binding sites; linker domain "L" (T_{610}-V_{668}); and C-terminal domain "CT" (H_{669}-R_{718}). The domains are drawn to scale [Figure source: Hassan, Moriyama et al. 2009].

HCS Deficiency

Multiple carboxylase deficiency (MCD) is a rare autosomal recessive metabolic disease caused by defects of enzymes, either biotinidase (biotinidase deficiency, OMIM #253260) or holocarboxylase synthetase (HCS deficiency, OMIM 253270), in the biotin cycle [Sherwood, Saunders et al. 1982; Sweetman, Burri et al. 1985]. Patients with MCD present in the neonatal period or early infancy with metabolic acidosis, hyperammonemia, tachypnea, skin rash, feeding problems, hypotonia, seizures, developmental delay, alopecia, and coma [Tammachote, Janklat et al. 2010]. Diagnosis is suggested by urine organic acids analysis and confirmed by holocarboxylase synthetase enzyme assay or DNA mutation analysis [Tammachote, Janklat et al. 2010]. As little as 10 milligrams per day of oral biotin usually improve clinical symptoms [Suormala, Fowler et al. 1997; Wolf 2001]. Patients with HCS deficiency usually have the neonatal or early-onset form, which may be fatal even within hours of birth [Sweetman, Nyhan et al. 1982], while patients with biotinidase deficiency have the juvenile or lateonset form. However, the age of onset and phenotypes are highly variable. Reliable diagnosis of

either MCD types therefore requires enzyme activity or genetic analysis [Tammachote, Janklat et al. 2010].

Mutations and SNPs in HCS

A series of mutations have been identified in the HCS gene [Dupuis, Leon-Del-Rio et al. 1996; Yang, Aoki et al. 2001; Suzuki, Yang et al. 2005]; these mutations decrease HCS activity substantially and cause features such as exfoliative dermatitis, hypoglycemia, ketoacidosis, and reduced histone biotinylation [Balnave 1977; Wolf, Heard et al. 1985; Baumgartner and Suormala 1999; Kobza, Camporeale et al. 2005; Atamna, Newberry et al. 2007]. While the prevalence of HCS mutations is low in the general population [Suzuki, Yang et al. 2005], early diagnosis is critical for a good prognosis. Mutations that decrease the affinity of HCS for biotin are particularly responsive to treatment with pharmacological doses of biotin [Suzuki, Yang et al. 2005]. To date, there are at least 24 mutations reported in *HCS* (http://www.hgmd.cf.ac.uk, accessed June 2011).

In addition, greater than 2,500 SNPs have been reported for HCS in public databases (http://www.genecards.org/cgibin/carddisp.pl?gene=HLCS&snp=2572&rf=/home/genecards/current/website/carddisp.pl#snp, accessed May 2011). The biological importance of these polymorphisms is unknown.

HCS Catalytic Activity Assay *in vitro*

Bioactivity of HCS has been probed *in vitro* using p67, a propionyl CoA carboxylase (PCC)-based polypeptide and a well-established HCS substrate [Lamhonwah,

Quan et al. 1987; Leon-Del-Rio and Gravel 1994; Campeau and Gravel 2001] which comprises the 67 C-terminal amino acids in human PCC and contains the consensus MKM motif for biotin bimding [Lamhonwah, Quan et al. 1987; Campeau and Gravel 2001].

The K_m Mutant Theory of HCS

Burri et al. characterized HCS activity in cultured cells from seven patients with considerable differences in disease severity [Burri, Sweetman et al. 1985]. They showed that the V_{max} values of patients' HCS were lower than the normal mean and were similar in all except one patient, and that the K_m values for biotin were elevated to various degrees. From their clinical and kinetic observations, they concluded that onset of the disease and its responsiveness to biotin administration is governed by the degree of abnormality in the K_m of HCS. Suormala et al. investigated the effects of biotin concentration on carboxylase activities in fibroblasts in five HCS-deficient patients [Suormala, Fowler et al. 1997]. The carboxylase activities of all patients normalized when the biotin concentration was increased to 10,000 nM. In carboxylase reactivation studies, although the kinetics of PCC activation varied greatly, all cells restored their PCC activity to the normal or nearly normal level (>87%). Reactivation of PCC activity in relation to time and biotin concentration correlated well with the severity and age at onset of the illness in four patients. These results were consistent with the original K_m mutant theory proposed by Burri et al. [Burri, Sweetman et al. 1985].

An expression study demonstrated that the K_m of the Val550Met HCS mutant was higher than, but the K_m of the Leu237Pro-mutant was the same as, that of the wild-type

enzyme [Aoki, Suzuki et al. 1997]. Thus, Sakamoto et al. questioned whether the K_m mutant theory could be applied to all patients with HCS deficiency, and proposed that not only the K_m of HCS for biotin, but also the V_{max} was an important factor in determining the severity of symptoms and their responsiveness to biotin therapy [Aoki, Suzuki et al. 1997].

The K_m values for biotin of the Gly581Ser and delThr610 mutant proteins were 45 and 3 times higher, respectively, than the wild-type value [Sakamoto, Suzuki et al. 1999]. Previous studies [Aoki, Suzuki et al. 1997] suggested that mutations within the homologous region (aa 448-701) to BirA result in elevated K_m values (K_m mutants). The mutations Arg183Pro, Leu216Arg, Leu237Pro, Val333Glu, and Val363Asp were located outside the homologous region (non- K_m mutants). The K_m values of these mutants differed only slightly from that of the wild type, whereas the V_{max} values were extremely lower than that of the wild type [Sakamoto, Suzuki et al. 1999]. The result suggested that non- K_m mutants can be a cause of biotin responsive HCS deficiency, thereby their hypothesis was confirmed [Aoki, Suzuki et al. 1997].

V_{max} Mutants of HCS

The V_{max} mutants group of mutations, namely Arg183Pro, Leu216Arg and Leu237Pro, map outside of the conserved catalytic core of HCS to the N-terminal region. Patients with these mutant HCS alleles vary in their responsiveness to biotin treatment. Although the N-terminal region has been implicated in catalysis [Campeau and Gravel 2001], the precise role played by this extension is poorly understood. These mutations have provided an insight into the structure and function relationships of HCS.

Interestingly, alignment of the BPL sequences from human, dog, horse, mouse, rat, chicken, Danio and Fugu show that the N-terminal extension is conserved amongst these vertebrate species. Furthermore, the equivalent amino acids at the above positions are invariant between these species, again highlighting their importance [Pendini, Bailey et al. 2008].

The Importance of HCS N-terminal Domain for its Catalytic Activity

The importance of the domain of HCS outside the homologous region (aa 448-701) to BirA for enzymatic activity was examined in the expression study with HCS deletion mutants by Hiratsuka et al. [Hiratsuka, Sakamoto et al. 1998]. The N-terminal amino acid region up to Ile117 had a much less effect on the activity than the amino acid sequence starting between Ile117 and Met234.

The Physiologic Biotin Concentration and HCS Catalytic Activity

The physiologic biotin concentration of human serum was estimated to be 1-3 nM and that of liver 0.5-4 nM [Baker 1985]. More recently, the serum biotin level was estimated to be about 0.24 nM [Mock, Lankford et al. 1995]. These concentrations are lower than the Km values of wild-type HCS that were measured in cultured fibroblasts [Burri, Sweetman et al. 1985; Suzuki, Aoki et al. 1996] or lymphoblasts [Burri, Sweetman et al. 1985], using CCP or PCC as a substrate. Therefore, the mutant HCS proteins harboring the Leu216Arg, Arg183Pro, Leu237Pro, Val333Glu, or Val363Asp would not show full activity in the physiologic state. Biotin treatment increases the biotin concentration in serum up to several hundred nanomolars [Burri, Sweetman et al.

1985; Suormala, Fowler et al. 1997], thereby helping to increase the velocity of biotinylation in the cells.

The Difference in the Mechanism of Biotin Attachment to Carboxylases and Histones

HCS was found to be a constituent of nuclei, in addition to the cytoplasm of cells, suggesting that HCS could be responsible for biotinylating histones in vivo [Cronan 2001]. However, as histones do not contain the biotin binding motif as seen in carboxylases, the mechanism and specificity of biotin attachment to histones might be expected to be significantly different [Healy, McDonald et al.].

III. Biotinidase (BTD)

Biotinidase belongs to the nitrilase superfamily of enzymes, which consists of 12 families of amidases, **N**-acyltransferases and nitrilases [Brenner 2002]. Some members of the nitrilase superfamily (vanins-1, -2 and -3) share significant sequence similarities with BTD [Maras, Barra et al. 1999]. Human BTD gene localizes to chromosome 3p25 and its structure has been determined [Cole Knight, Reynolds et al. 1998]. The human *BTD* gene contains four exons: $A_{125\text{-}44}$, $B_{45\text{-}309}$, $C_{310\text{-}459}$ and $D_{460\text{-}1961}$, and they span at least 44 kb and encode a protein of 543 amino acids [Stanley, Hymes et al. 2004]. The mature enzyme is encoded by exons B through D. The coding region of BTD has two in-frame start codons, both of which might initiate translation [Stanley, Hymes et al. 2004].

The cellular distribution of BTD is controversial. While the existence of BTD acitivty in microsomes and mitochondria is accepted universally [Pispa 1965; Garganta and Wolf 1990; Nilsson and Ronge 1992], the presence of BTD in nuclei is less certain [Zempleni, Hassan et al. 2008].

The classical role of BTD in metabolism is to hydrolyze biocytin (biotinyl- ε -lysine) generated in the breakdown of biotin-dependent carboxylases [Hymes, Fleischhauer et al. 1995]. Thus, free biotin is released and recycled in holocarboxylase synthesis and used in other processes [Camporeale and Zempleni 2006]. The role of BDT in biotinylation of histones has been demonstrated based on *in vitro* studies with purified histones [Hymes, Fleischhauer et al. 1995]. It was proposed that BTD might mediate the binding of biotin to histones by the hydrolysis of biocytin forming a biotinyl-thioester intermediate (cysteine-bound biotin) near its active site. Thus, biotin is bound to the biotinyl moiety from the cysteine residue and transfer to the ε-amino group of the lysine in histones [Hymes, Fleischhauer et al. 1995; Hymes and Wolf 1999]. The Km value for hydrolysis of biocytin by biotinidase is in the micromolar range and hydrolysis occurs optimally at pH 5.5–6, with a precipitous decrease in activity above pH 7. On the other hand, biotinylation of histones by biotinidase occurs at physiological pH and concentrations of biocytin (nanomolar range) [Hymes, Fleischhauer et al. 1995].

Evidence has been provided that BTD might catalyze debiotinylation of histones [Ballard, Wolff et al. 2002]. It was proposed that variables such as microenviroment in chromatin, posttranscriptional modifications and alternative splicing might determine whether BTD acts as a biotinyl histone transferase or histone debiotinylase [Zempleni 2005]. For example covalent modifications such as glycosylation might influence the

enzyme activity to biotinylate or debiotinylate histones [Cole, Reynolds et al. 1994; Ballard, Wolff et al. 2002]. In the same way, the presence of cofactors such as high concentrations of biocytin might favor and increase the rate of biotinylation of histones [Ballard, Wolff et al. 2002].

IV. Histone Biotinylation

Hisotnes and Chromatin

Histones are small basic proteins (11-22 kDa) involved in the folding of the DNA into chromatin. They are modified by the covalently binding of biotin to lysine residues by holocarboxylase synthetase [Wolffe 1998; Stanley, Griffin et al. 2001]. There are five major classes of histones in chromatin: H1, H2A, H2B, H3 and H4 [Wolffe 1998]. Histones H2A, H2B, H3 and H4 form an octamer of core histones with one H3-H3-H4-H4 tetramer and two H2A-H2B dimers while histone H1 is the linker histone bound to the DNA in between two nucleosomes (**Fig. 6**) [Kothapalli, Camporeale et al. 2005]. Importantly, histones are positively charged proteins due to the presence of a large number of lysine and arginine residues (20-25% of the amino acids). The electrostatic interaction between the negative charge of DNA and the positive charge of histones stabilizes the formation of the nucleosomal core particle [Wolffe 1998].

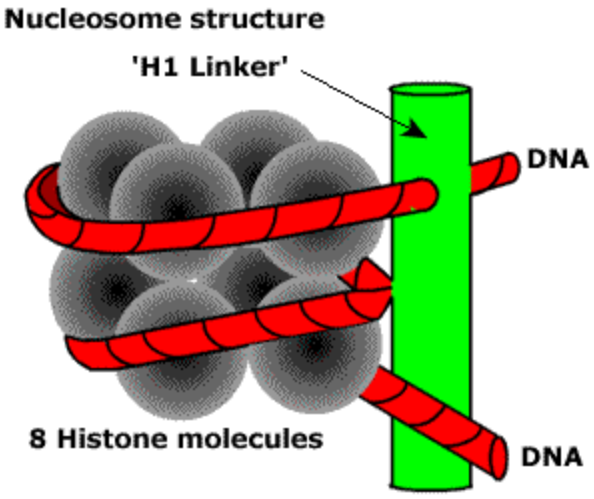


Figure 6. Nucleosome structure. Histone H1 and octamer.

Covalent Histone Modifications

Histones are target for reversible covalent modifications such as acetylation, methylation, phosporylation, poly (ADP-ribosylation) and biotinylation. These modifications have distinct functions in gene expression, chromatin remodeling, transcriptional regulation, and DNA repair [Turner 2000; Moore and Krebs 2004; Narang, Dumas et al. 2004; Khan and Krishnamurthy 2005]. For example, acetylation of lysine residues has been associated with transcriptional activity of genes [Bannister, Schneider et al. 2002]. Methylation may be associated with transcriptional activation or repression. Phosphorylation and poly (ADP-ribosylation) are associated with signaling and DNA repair [Peters, Griffin et al. 2002].

Histone Biotinylation

In previous studies, 11 biotinylation sites in histones have been identified: K9, K13, K125, K127 and K129 in histone H2A [Chew, Camporeale et al. 2006]; K4, K9, K18 and perhaps K23 in histone H3 [Kobza, Sarath et al. 2008]; and K8 and K12 in histone H4 [Camporeale, Shubert et al. 2004] (**Fig. 7** [Ho and Zempleni 2009]).

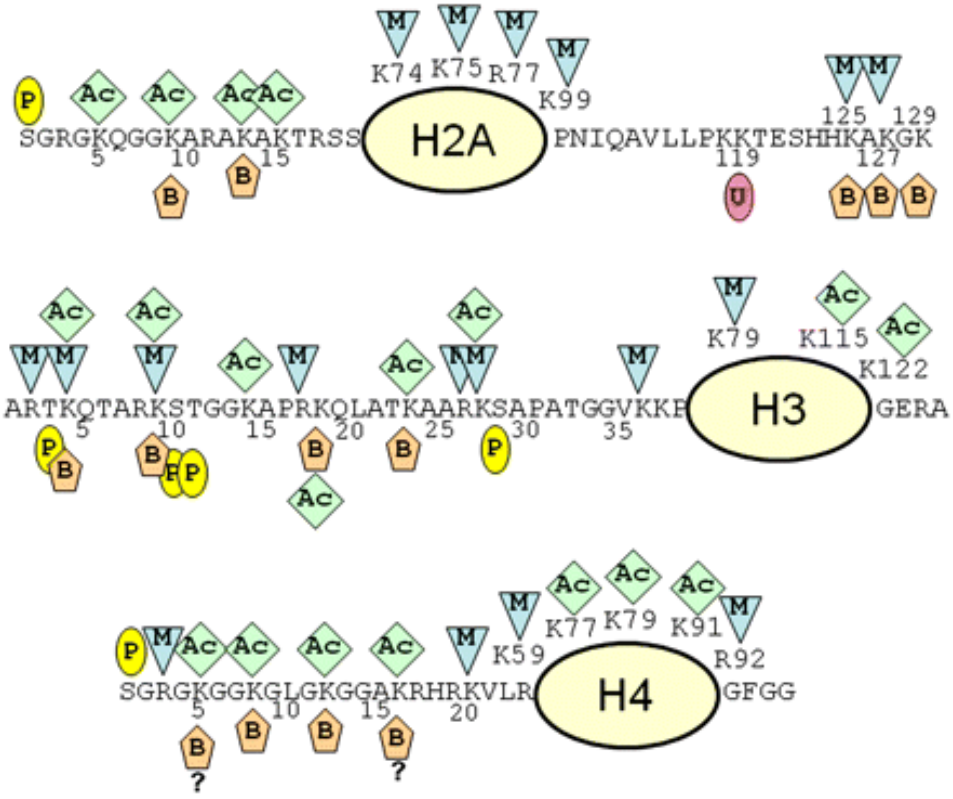


Figure 7. Modification marks in histones H2A, H3, and H4 ([Figure source: Ho and Zempleni 2009]). Abbreviations: Ac, acetate; B, biotin; M, methyl; P, phosphate; U, ubiquitin [Jenuwein and Allis 2001; Fischle, Wang et al. 2003; Camporeale, Shubert et al. 2004; Kobza, Camporeale et al. 2005; Chew, Camporeale et al. 2006; Chew, Raza et al. 2006; Kouzarides and Berger 2007; Kobza, Sarath et al. 2008]. Marks labeled with "?" are based on preliminary observations and await confirmation [Camporeale, Shubert et al. 2004; Chew, Raza et al. 2006].

Biotinylation of histones plays an important role in the cellular response to DNA damage [Kothapalli, Sarath et al. 2005], cell proliferation [Stanley, Griffin et al. 2001], and mitotic condensation of chromatin [Kothapalli and Zempleni 2005]. In fact, H4K12bio has been associated with gene silencing [Camporeale, Oommen et al. 2007] and repression of transponsable elements [Chew, West et al. 2008].

The Role of HCS in Biotinylation of Histones

The important role of HCS in biotinylation of histones has been confirmed in various studies [Leon-Del-Rio, Leclerc et al. 1995; Camporeale, Giordano et al. 2006]. Camporeale et al. compared the ability of SMVT mutants and HCS-deficient *Drosophila melanogaster* to biotinylate histones. The results demonstrated that HCS knockdown is associated with a decrease in biotinylation of histones due to the low biotin-ligase activity, rather than the low levels of biotin in the cells [Leon-Del-Rio, Leclerc et al. 1995]. Importantly, Bao et al. recently reported that HCS interacts physically with histone H3 to mediate binding to chromatin and subsequent biotinylation of K9 and K18 in histone H3 [Bao, Pestinger et al. 2011].

MATERIALS AND METHODS

Candidate SNPs selection

As of May 7, 2011, 2572 SNPs in HCS gene have been reported on "GeneCards" website (http://www.genecards.org/cgibin/carddisp.pl?gene=HLCS&snp=2572&rf=/home/genecards/current/website/carddisp.pl#snp). The SNPs in the coding region of HCS gene were detected out of these. Candidate SNPs were then limited to those which cause amino acid changes. Furthermore, candidate SNPs were limited to those which were suspected to cause HCS conformational changes, e.g., substitution of basic for hydrophobic amino acid, for further biochemical analysis.

Recombinant HCS

A clone of full-length human HCS was provided by Yoichi Suzuki (Tohoku University, Sendai, Japan) [18]. *EcoRI* and *XhoI* restriction sites were inserted by PCR, using forward primer 5'-GTCCGAATTCGGGGAAGATAGACTCCACATGGATAATG-3' and reverse primer 5'-ATTTCTCGAGCCCGCCGTTTGGGGAG-3'. The PCR product was digested with *EcoRI* and *XhoI* and cloned into vector pET41a(+) (Novagen, Madison, WI), fusing glutathione S-transferase (GST), S·tag, and a 6x his·tag to the N-terminus of HCS; this construct also contains a 6x his·tag at the C-terminus of HCS. The plasmid was named "HCS-pET41a(+)" and codes for a fusion protein of 114.6 kDa; its identity was verified by sequencing [Bao, Pestinger et al. 2011].

Site-directed mutagenesis

5 kinds of different SNPs in HCS gene were chosen for further analysis. HCS-pET41a(+) was digested with *EcoRI* and *XhoI* and subcloned into vector pBluescript II SK (+) (Stratagene; Wilmington, DE). The plasmid was named "HCS- pBluescript II SK (+)". Each mutation was introduced to HCS-pBluescript II SK (+) using GeneTailor™ Site-Directed Mutagenesis System (Invitrogen; Carlsbad, CA). Each plasmid was then digested with *EcoRI* and *XhoI* and subcloned into vector pET41a(+).

Expression and purification of recombinant HCS

ArcticExpress (DE3) Competent Cells (Agilent Technologies; Santa Clara, CA) transformed with HCS-pET41a(+) [Bao, Pestinger et al. 2011] were cultured in LB Broth Medium with 20 μg/ml of gentamycin and 50 μg/ml of kanamycin, and incubated overnight at 37°C. Bacteria were allowed to grow until an OD600 of 0.5- 1.0 was reached. Protein expression was induced adding Isopropyl-beta-D-thiogalactopyranosid (IPTG) at a final concentration of 1mM, and incubated at 12ºC for 24 hours. Cells were centrifuged at 4ºC (3,000 g for 30 minutes) and precipitated cells were resuspended in PBS with protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Samples were sonicated on ice (Branson 250 Digital Sonifier, Danbury, CT) three times for 10-second burst and alternated with 10-second resting periods on ice. The lysate were centrifuged at 10,000 g for 10 minutes at 4ºC. HCS fusion protein were purified by GSTrap™ FF Columns (GE Healthcare; Piscataway, NJ) on ÄKTA™ protein purification system (GE Healthcare). HCS in column fraction were identified by gel electrophoresis followed by

coomassie blue staining and western blot using an antibody to the C-terminus in human HCS.

Expression and purification of recombinant p67

The polypeptide p67 is frequently used to confirm biological activity of HCS. It is the C-terminal 67 amino acid biotin carboxyl carrier (BCC) domain of the propionyl-CoA carboxylase (PCC) α subunit (Leu637-Glu703). Rosetta™ 2(DE3) Competent Cells (EMD Chemicals; Gibbstown, NJ) transformed with p67- pET30 [Kobza, Sarath et al. 2008] were cultured in LB Broth Medium with 15 µl of kanamacyn, and incubated overnight at 37°C. Cells were allowed to grow until an OD600 of 0.5 - 0.6 was reached. p67 expression was induced by IPTG 1 mM final concentration and incubated for 16 hours at 37ºC. After incubation, samples were centrifugated at 3,000 g for 30 minutes and purified using His Trap FF Columns (GE Healthcare) on an ÄKTA™ protein purification system (GE Healthcare). Because BirA ligase in Escherichia coli is known to biotinylate some of the recombinant p67, the biotinylated fraction of p67 was removed using avidin columns.

Normalization of the HCS constructs

Purified proteins of wild-type HCS and its variants were mixed with NuPAGE® LDS Sample Buffer (4X) (Invitrogen), boiled at 95 ºC for 10 minutes, and loaded into NuPAGE® Novex 4-12% Bis-Tris Gel (Invitrogen). Relative concentrations of each

recombinant HCS (rHCS) construct were normalized by staining with coomassie blue and by western blot using an antibody to the C-terminus in human HCS.

HCS catalytic activity assay using p67 as substrate

Bioactivity of the purified HCS (50 nM) was measured using 50 µl of a mix containing 75 mM Tris- Acetate (pH 7.5), 7.5 mM ATP, 0.3 mM dithiothreitol (DTT), 45 mM $MgCl_2$, 45 µM p67 and graded concentration of biotin (0, 1, 3, 6, 12 and 24 µM). The mix was incubated at 37 ºC for 3 hours. 50 µl of tricine loading buffer was added to the mix. The mix (100 µl) was boiled at 95 ºC for 10 minutes. 15 µl of the sample was loaded and run on a 16% tricine gel. The gel was transferred onto the PVDF membrane. The membrane was dipped in PBS to remove traces of transfer buffer, incubated with Odyssey blocking buffer (Licor; Lincoln, NE) at room temperature for 1 hour, and washed with 0.1% T-PBS (PBS containing 0.1% Tween 20) at room temperature 3 times - 5 minutes each - with gentle rotation. The membrane was then transferred to a black tray containing 2.5 µL of the stock solution of IRDye 800CW Streptavidin (Licor; 2.4 moles of IRDye 800 CW/1 mole of streptavidin) in 20 mL of 0.1% TPBS and incubated for 1 hour at room temperature. The membrane was washed with 0.1% TPBS containing 5 M NaCl at room temperature 4 times - 5 minutes each - with gentle rotation. The membrane was scanned to detect the probed p67-bound biotin by measuring infrared absorbance of IRDye 800CW Streptavidin at 800 nm (channel 800CW) in the Odyssey imaging system. Non-linear regression analysis was used to calculate K_m and V_{max} of

HCS for biotin using the software "GraphPad Prism 5.00" (GraphPad Software; La Jolla, CA).

Statistical Analysis

Homogeneous variances were identified by using Bartlett's test. Significance of differences was tested by one-way ANOVA. Fisher's Protected Least Significant Difference procedure was used for posthoc testing. StatView 5.0.1 (SAS Institute; Cary, NC) was used to perform all calculations. Differences were considered significant if $\mathbf{P} < 0.05$. Data were expressed as mean ± SD.

RESULTS

Bioinformatics

It turned out that there are 14 HCS variants overall in the coding region of HCS in GeneCards database. 6 variants of them do not cause amino acid changes (group 1). 4 variants of them cause amino acid changes, but the side chains have exactly the same characteristics before and after the substitutions (group 2): the substitution of aspartate for glutamate (both have hydrophilic and negatively charged R groups), the substitution of histidine for arginine (both have hydrophilic and positively charged R groups) and the substitution of leucine or isoleucine for valine (SNP ID: rs61731502; both have hydrophobic R groups). The rest 4 variants cause amino acid substitutions by which the characteristics of the side chains also change (group 3). The variant rs61732502

substitutes phenylalanine (hydrophobic and aromatic R groups) for valine (hydrophobic and aliphatic R groups), the variant rs28934602 substitutes arginine (hydrophilic and positively charged R groups) for leucine (hydrophobic and neutral R groups), the variant rs75867009 substitutes arginine for glycine (hydrophobic and positively charged R groups) and the variant rs79373682 substitutes arginine for glutamine (hydrophobic and positively charged R groups).

The 5 variants below – one from group 2 and all four variants from group 3 - were chosen for further catalytic analysis (**Table 1**).

Table 1. HCS variants

SNP ID	nucleotide substitution	amino acid substitution	amino acid side chains' characteristics	HCS domain
rs61732502	G286T	V96F	• Both are hydrophobic. • aliphatic → aromatic	N-terminal
rs61732502	G286C	V96L	Both are hydrophobic.	N-terminal
rs28934602	T647G	L216R	hydrophobic and neutral → hydrophilic and basic (positively charged)	N-terminal
rs75867009	G1528A	G510R	• neutral → basic (positively charged) • Both are hydrophilic. • Arginine is much bulkier than Glycine.	central
rs79373682	A2096G	Q699R	• neutral → basic (positively charged) • Both are hydrophilic.	C-terminal

Recombinant HCS and p67

After recombinant proteins of wild-type HCS and the variants shown above were produced, relative concentrations of each recombinant HCS (rHCS) construct were normalized by staining with coomassie blue (Fig. 8 (a)) and by western blot using an

antibody to the C-terminus in human HCS (Fig. 8 (b)). Recombinant p67 is biotinylated by BirA in *E. coli* and therefore requires affinity purification before use. When recombinant p67 was purified by using avidin columns, the vast majority of biotinylated p67 was removed. For all assays described below, biotin-depleted p67 was used.

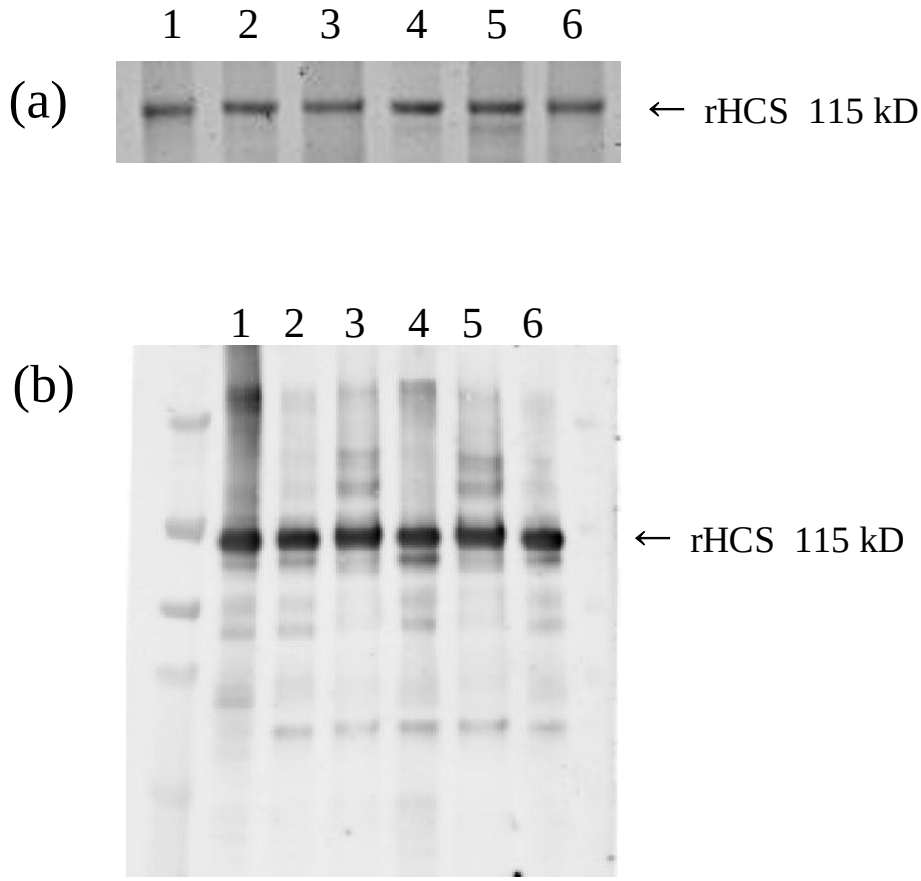


Figure 8. Normalization of rHLCS concentrations for enzyme kinetics studies. Wild-type HLCS (WT) and variants were stained with coomassie blue (panel a) and probed with anti-HLCS (panel b). 1: WT, 2: V96F, 3: V96L, 4: L216R, 5: G510R, 6: Q699R.

Dose-response studies in HCS catalytic activity assay using p67 as substrate

A titration of wild-type rHCS was conducted with overabundant amount of p67 (45 μM) and biotin (100 μM) to determine a concentration where enzyme concentration is proportional to the amount of substrate biotinylation when substrate and coenzyme availability are not limiting factors in this HCS catalytic assay. The amount of biotinylated p67 in this assay increased linearly up to a concentration of rHCS of 60 nM, and leveled off thereafter. Subsequent experiments were conducted using 50 nM HCS to ensure assay linearity.

Then p67 was titrated with an overabundant amount of biotin (100 μM) to determine a concentration where substrate availability is no longer a limiting factor in this assay. A linear dose-response was obtained for up to 20 μM p67, while p67 was no longer rate limiting beyond that concentration. For subsequent experiments, p67 was used at a concentration of 45 μM.

Kinetics analysis of p67 biotinylation by wild-type rHCS and its variants

We determined the kinetic properties of the wild-type and five rHCS variants using p67 as substrate. **Table 2**, **Fig. 9 and Fig. 10** show K_m values for biotin and $\mathbf{V}_{max}$ values of the rHCS constructs. Note that the activity of L216R was too low to allow for meaningful analysis of K_m. All these V_{max} and K_m values were homogeneous. **Fig. 11 (a) – (e)** are the graphs of initial velocity depending on biotin concentration. Three variants, V96F, V96L and L216R, are within the N-terminal domain (aa1-446). The variant Gly510Arg is within the central domain (aa471-575). And the variant Q699R is within the C-terminal domain (aa669-718).

Three variants, V96F, L216R and G510R, had significantly different V_{max} values compared with that of the wild type. The V_{max} values of V96F and G510R variants were decreased to a slight extent: 78% and 73%, respectively (percentage of the wild-type value), and the V_{max} value of L216R variant was markedly decreased: 6%.

Only the Q699R variant had significantly different K_m value compared with that of the wild type. The K_m values of this mutant enzyme for biotin were higher than that of the wild type by 57%.

Table 2. Vmax and Km values of wild-type rHCS and its variants

	WT	V96F	V96L	L216R	G510R	Q699R
Vmax (pmol biotin/(h*pmol HCS))	3.9±0.3	3.0±0.5	4.1±0.4	0.2±0.04	2.8±0.5	3.9±0.6
Vmax (% wild type)	100	78	106	6	73	101
Km (μM)	4.0±1.0	4.0±1.9	5.1±1.4	n.d. *	3.4±1.9	6.3±2.6
Km (fold over wild type)	1	1	1.27	n.d. *	0.84	1.57

* n.d.=not detectable. The activity of L216R was too low to allow for meaningful analysis of K_m.

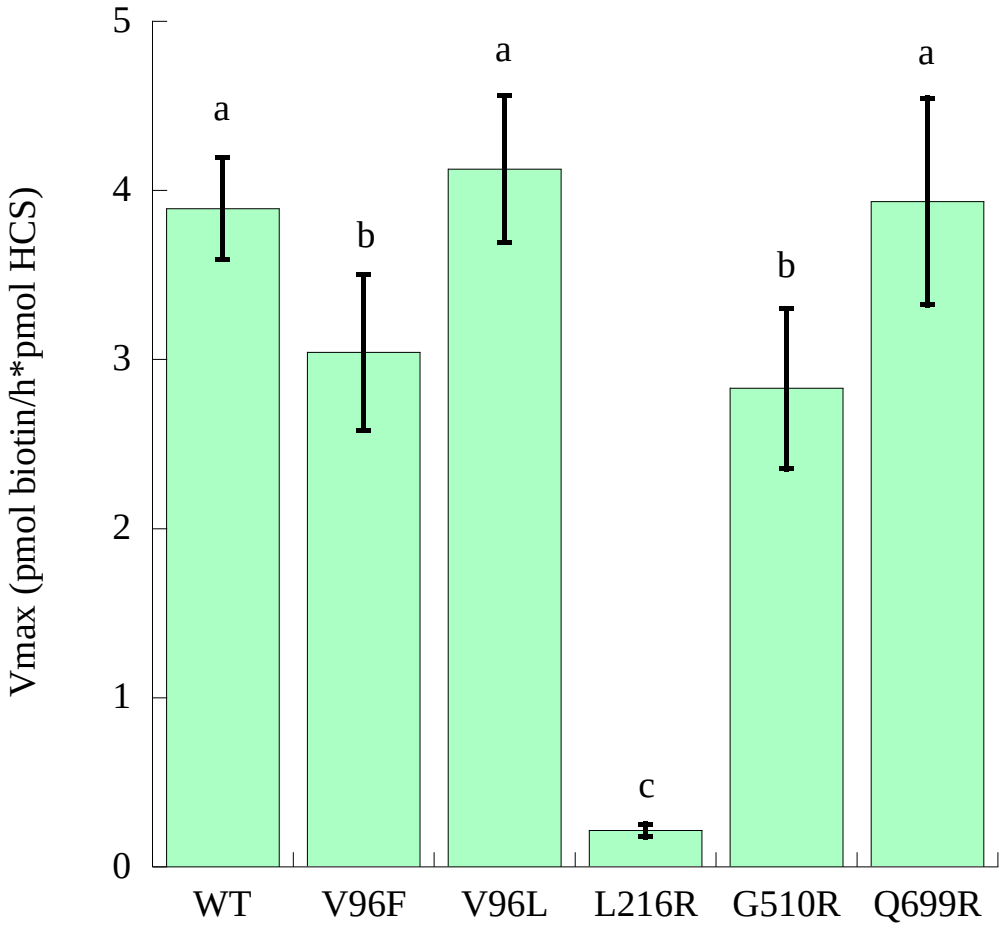


Figure 9. V_{max} **values of the wild-type rHCS and its variants.** rHCS was incubated with p67, biotin and cofactors for 3 hours; enzyme activity was measured by infrared spectroscopy. Bars without a common letter are significantly different (n=3, $P<0.05$).

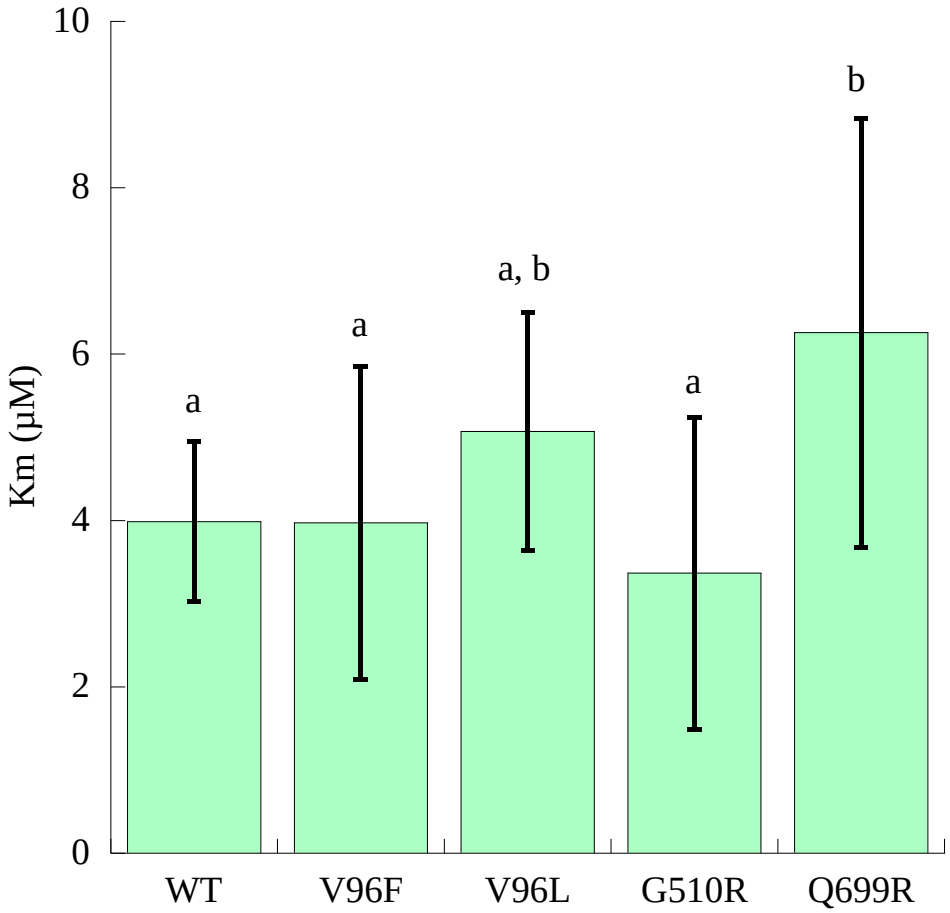
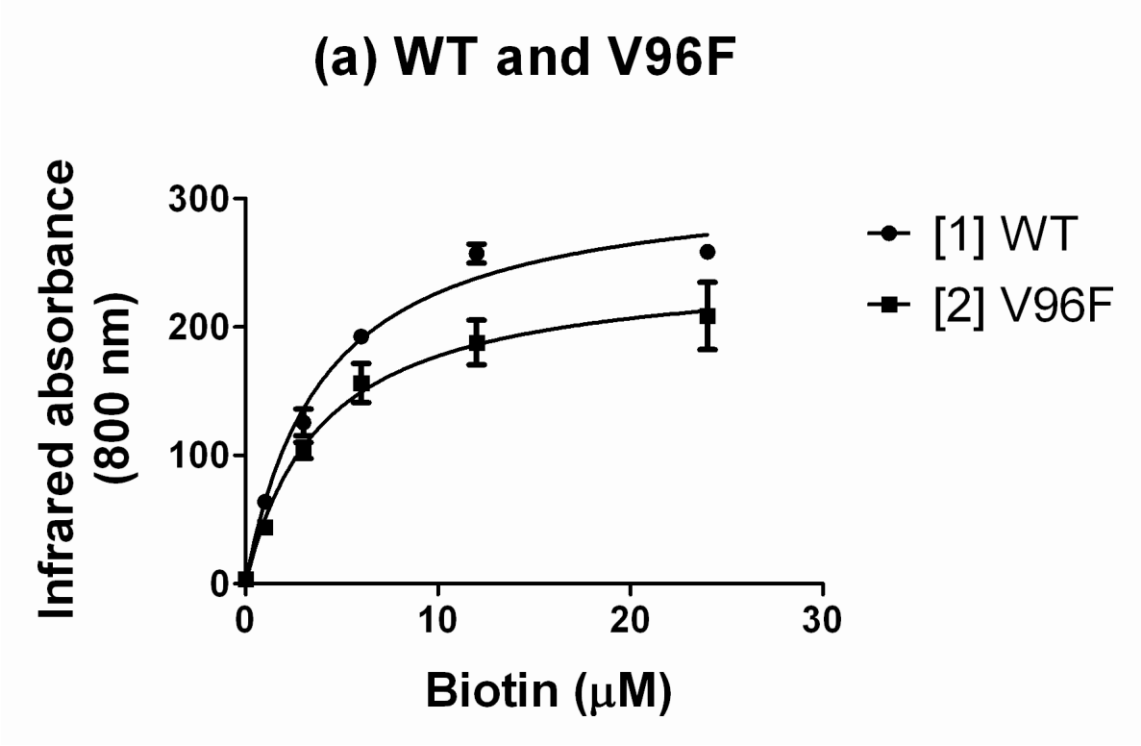
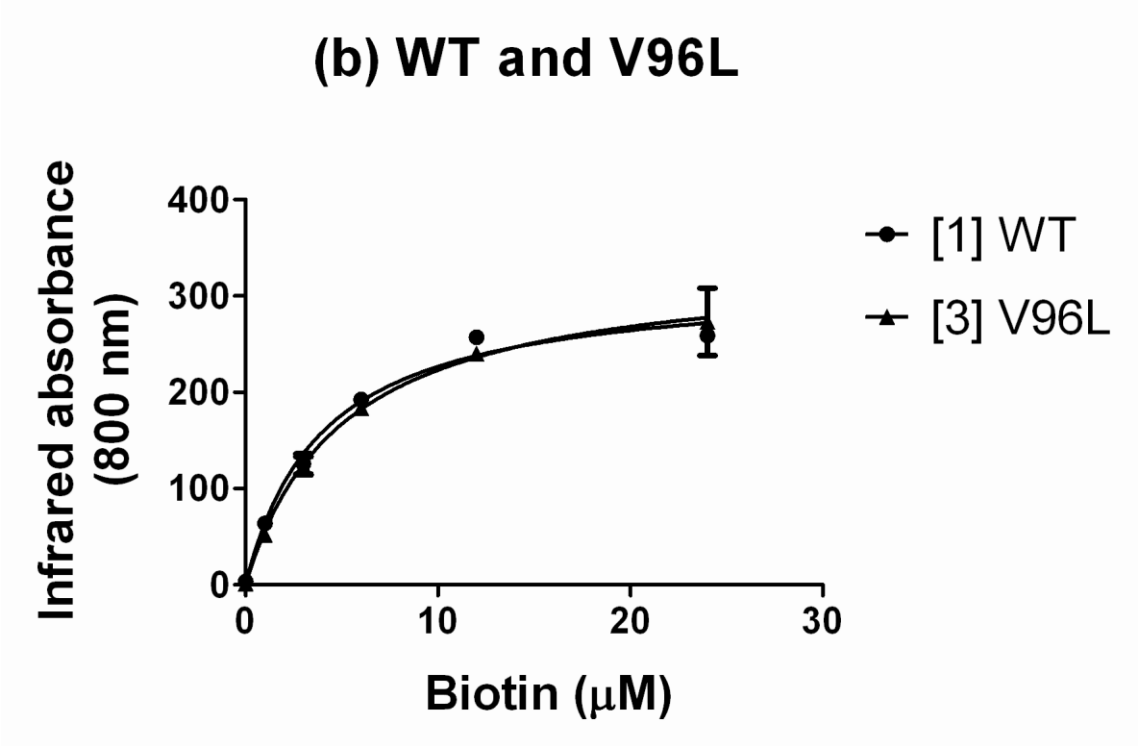
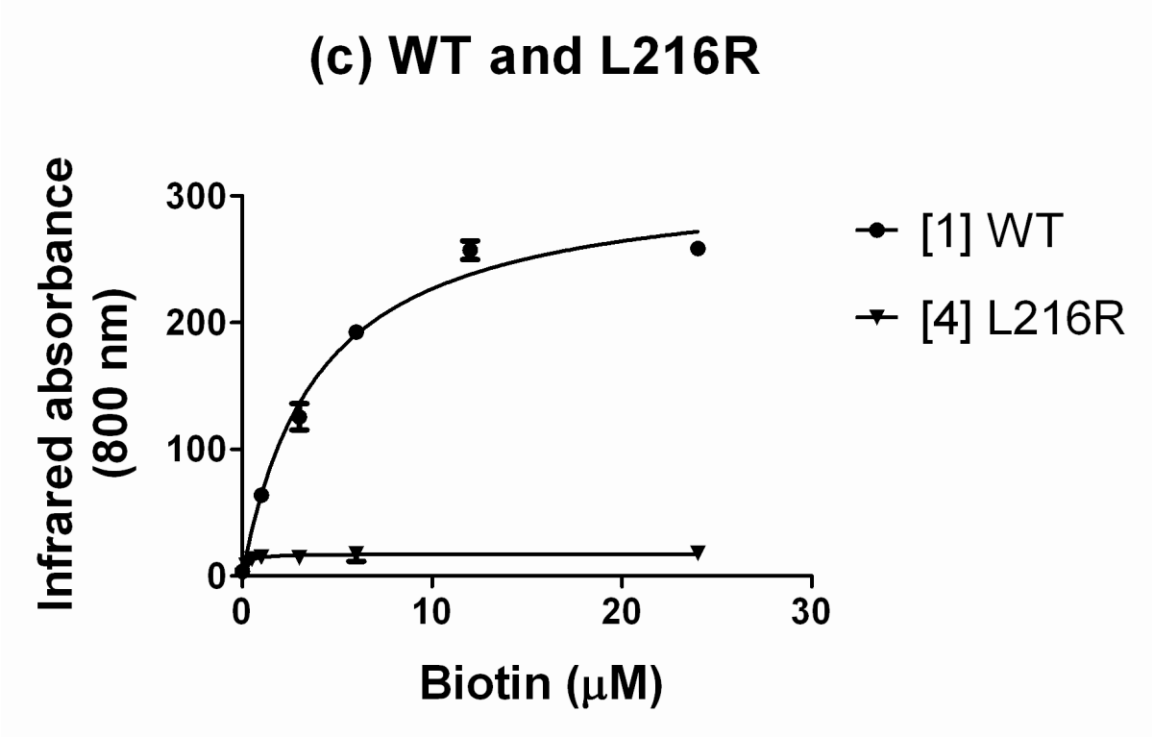
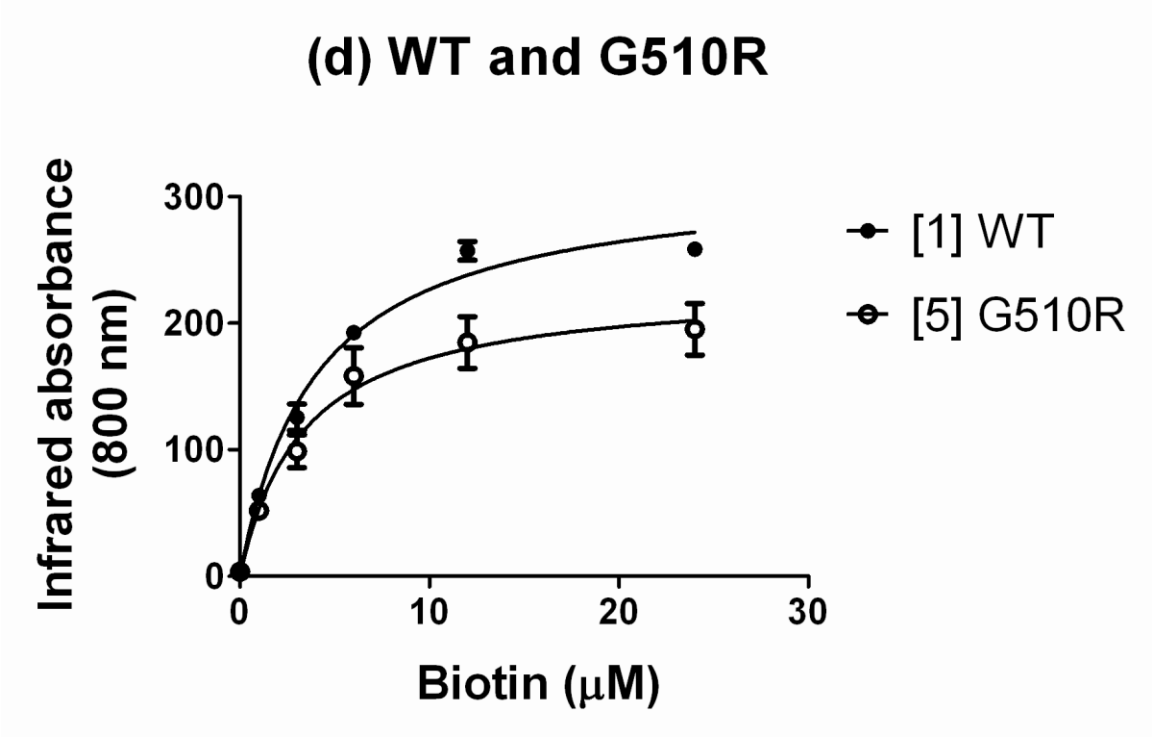


Figure 10. K_m **values of the wild-type rHCS and its variants.** rHCS was incubated with p67, biotin and cofactors for 3 hours; enzyme activity was measured by infrared spectroscopy. Note that the activity of L216R was too low to allow for meaningful analysis of K_m. Bars without a common letter are significantly different (n=3, $P<0.05$).


(a) WT and V96F
Infrared absorbance (800 nm)
300
200
100
0
0
10
20
30
Biotin (μM)
[1] WT
[2] V96F


(b) WT and V96L
Infrared absorbance (800 nm)
400
300
200
100
0
0
10
20
30
Biotin (μM)
[1] WT
[3] V96L


(c) WT and L216R
Infrared absorbance (800 nm)
300
200
100
0
0
10
20
30
Biotin (μM)
[1] WT
[4] L216R


(d) WT and G510R
Infrared absorbance (800 nm)
300
200
100
0
0
10
20
30
Biotin (μM)
[1] WT
[5] G510R

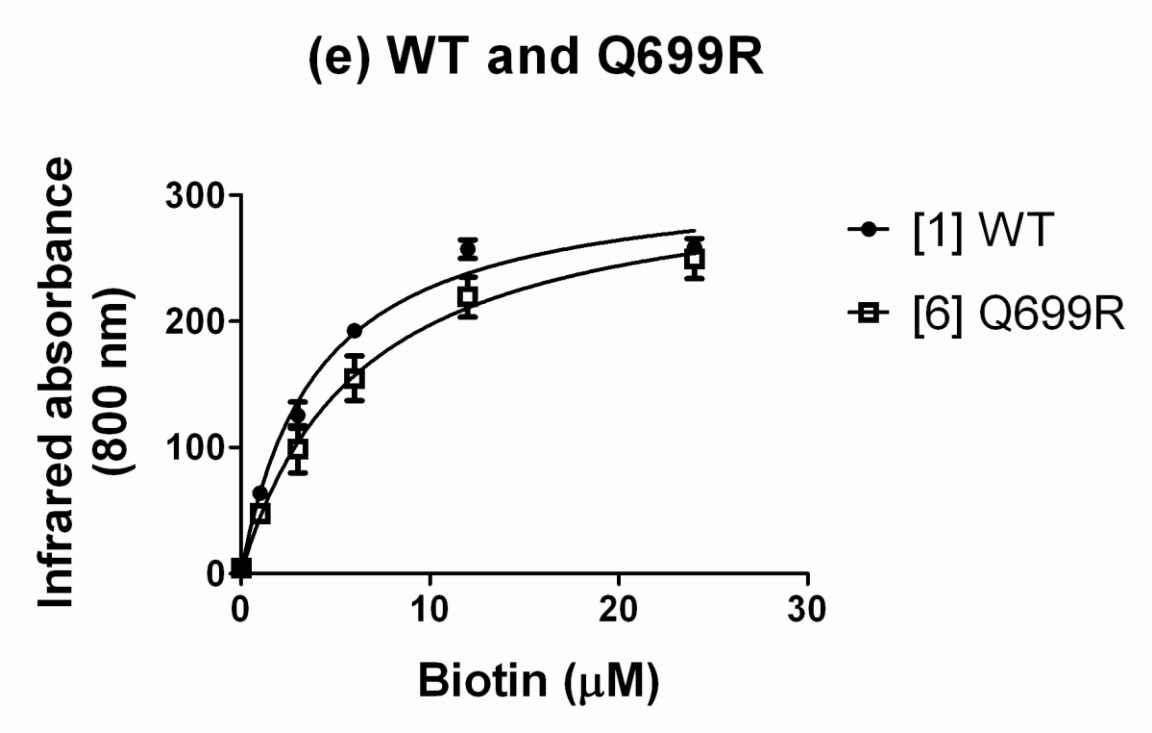


Fig 11 (a)-(e). Non-linear regression analysis of HLCS variants V96F (panel a), V96L (b), L216R (c), G510R (d), and Q699R (e) compared with wild-type HLCS. Data points were fitted by using the Michaelis-Menten equation (N=3 independent analyses for each HCS variant). Abbreviations: WT = wild type.

SNPs that affect the catalytic activity of HCS

The variant V96F had 78% V_{max} and 100% K_m of that of the wild type, suggesting that this variant's affinity for biotin was the same level as wild type, and the activity of this enzyme was compromised by 22% but not restored with higher concentrations of biotin.

The variant V96L had 106% V_{max} and 127% K_m of that of the wild type, suggesting that the affinity for biotin and the activity of this variant were the same level as wild type statistically.

The variant L216R had as low as 6% V_{max} of that of the wild type (Km value was not available), suggesting that the activity of this enzyme was compromised by 94% but not restored with higher concentrations of biotin.

The variant G510R had 73% V_{max} and 84% K_m of that of the wild type, suggesting that this variant's affinity for biotin was the same level as wild type statistically, and the activity of this enzyme was compromised by 27% but not restored with higher concentrations of biotin.

The variant Q699R had 101% V_{max} and 157% K_m of that of the wild type, suggesting that this variant's affinity for biotin was lower by 57% than wild type, and the activity of this enzyme was the same level as wild-type enzyme because it was restored with higher concentrations of biotin.

The concentration of biotin needed to achieve normal substrate biotinylation by HCS variants compared with wild-type HCS

Of the 5 variants studied here, V96L variant showed the same affinity for biotin and the same activity as wild-type enzyme. The activity of the variants V96F, L216R and G510R was compromised but not restored with higher concentrations of biotin.

In contrast, the affinity of variant Q699R for biotin was significantly lower than that of wild type HCS (K_m: 1.57 times that of wild type) and its V_{max} could be restored to that of wild-type HCS by biotin supplementation (Fig. 11-(e)).

Our knowledge of the intracellular concentrations of biotin in mammalian tissues is rather limited. Evidence suggests that the concentrations of biotin in liver and kidney is about 3.3 μmol/kg and 4.5 μmol/kg, respectively, in pigs fed a normal diet containing

200 µg biotin/kg [Kopinski, 1989]. Likewise, the concentration of biotin is about 500—900 fmol per cell in human embryonic palatal mesenchymal cells in culture [Takechi, 2008], which translates into a concentration of about 6.8 µmol/L assuming a cellular volume of 0.3 nL [Nibbering, 1990]. The tissue concentration of free biotin might be lower than that of protein-bound biotin [Rathman, 2002], but this might be offset by local accumulation of free biotin in HLCS-rich microdomains. Notwithstanding the uncertainties with regard to the true concentrations of biotin in human tissues and cellular compartments, we are reasonably confident that the K_m values reported for HCS in this study are similar to the concentrations of biotin in human tissues. If so, biotin supplementation might benefit individuals homozygous for the HLCS variant Q699R.

DISCUSSION

The two objectives of this study were (1) to identify HCS SNPs that affect the catalytic activity of HCS and (2) to determine if higher concentration of biotin could restore normal substrate biotinylation by HCS variants compared with wild-type HCS.

There have been multiple reported mutations in the HCS gene that give rise to MCD (reviewed in Bailey et al. 2008 [Pendini, Bailey et al. 2008]; summarized in **Table 3**). The severity of disease varies greatly with the genotype of each individual. Broadly, the mutations can be classified into two groups. Firstly the K_m mutants are amino acid substitutions that result in an enzyme with decreased affinity for biotin. Patients bearing these mutant HCS respond well to oral administration of pharmacological doses of biotin.

Secondly, the V_{max} mutants are substitutions where the activity of the enzyme is compromised but not restored with higher concentrations of biotin.

According to these mutant theories, V96F, L216R and G510R variants are V_{max} mutants, and Q699R variant is a K_m mutant for the variants studied in this thesis. This observation agrees with the report by Sakamoto et al. [Sakamoto, Suzuki et al. 1999] that L216R is a V_{max} mutant, and with the report that most of the K_m mutants lie in the C-terminal catalytic portion of the enzyme [Pendini, Bailey et al. 2008]. To date there are no reports of an X-ray structure for human HCS, but recent studies reporting new structures of BPL from bacteria E. coli has provided powerful insights into the HCS structure.

Table 3. Biochemical characterisation of HCS missense mutations. [Table source: Bailey et al. 2008]

Mutation	Activity (% wildtype)	Km Biotin (fold over wildtype)
Wildtype	100	1
E42D	120	N.D.
R183P	1.7	0.6
L216R	0.3	1.4
L237P	1.2-4.3	0.4–1.2
V333E	2–10	1.5
R360S	22	N.D.
V363D	3.7	1.1
Y456C	0.2	N.D.
T462I	<10	N.D.
L470S	4.3	N.D.
R508W	34.5	23
V547G	3.4	N.D.
V550M	16.6	6.5
D571N	0.1	N.D.
G581S	<10	44.3
D634Y	12	N.D.

Amino acid substitutions in the catalytic region (residues 422–726) induce a 3–44-fold elevated K_m for biotin compared to wildtype HCS. N.D: Not determined.

Unlike mutations in the HCS gene, there have been no reports which focused on SNPs in the HCS gene to date. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs, which make up about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome. Many SNPs have no effect on cell function, but others could predispose people to disease or influence their response to a drug, etc. This is the first comprehensive study of the catalytic activity of HCS SNPs.

Of the 5 substitutions studied in this thesis, L216R has already been reported in the UniProt database (P50747). This allele was first reported as a mutation in the human HCS gene, and has subsequently appeared to occur at a high frequency in the Samoan and Cook Island populations [Morrone, Malvagia et al. 2002], which is probably the reason why this variant was determined as a SNP. As our data in this thesis shows, this allele results in markedly reduced enzyme activity. According to the report by Morrone et al., five of the seven homozygous patients with this variant died between age 3 days to 3 years. All patients presented within 24 hours of birth with severe acidosis. The two babies that did not receive biotin treatment died within seven days. Despite biotin therapy, three of the patients continued to show severe dermatological symptoms and recurrent septicaemia followed by metabolic decompensation. All died during one of these episodes [Morrone, Malvagia et al. 2002]. In contrast, a patient with this variant in the heterozygous form (L216R /V363D) showed good clinical response to biotin therapy (10–40 mg/day) [Dupuis, Campeau et al. 1999]. Biotin responsiveness was attributed to the presence of the biotin-responsive allele V363D. Patients homozygous for the L216R

variant have since been reported and, their response to biotin therapy has varied for some reason [Wilson, Myer et al. 2005]. Interestingly, a recent study demonstrated that this variant induced increased protein turnover thereby providing a partial explanation for the poor response to biotin therapy [Bailey, Ivanov et al. 2008]. Further crystallographic studies on the HCS structure will be required to fully understand how mutations distal from the active site of HCS have an effect on function.

Blast search against PDB databank returned prokaryotic biotin ligase (BirA) structures alignment to the central and C-terminal domains of the human HCS (alignment from around residues 460 to 710 aa), with approximately 31% sequence identity. A 3D modeling would give detailed information about the changed molecular interactions due to the substitution. However, modeling is only possible for the central and C-terminal domains (i.e. for G510R and Q699R), for which structural information is available while no human or eukaryotic biotin ligase structure is available to date.

HCS substitutions G510R and Q699R are located at the equivalent sites in the bacterial structures, indicating that substitution sites 510 and 699 are not in the biotin binding site and are located in the solvent exposed exterior of the protein (**Fig. 12** and **Fig. 13**). In the central domain of human HCS, the R508-S515 loop corresponds to one of the biotin-binding site (R118-S125) in BirA [Hassan, Moriyama et al. 2009]. However, substitution G510R does not yield any specific hydrogen bond interactions with the co-factor. In contrast, the mutations R508W and N511K have been reported to cause severe damage to the catalytic activity of HCS [Pendini, Bailey et al. 2008] (**Fig. 12)** because these sites are critical residues in the loop covering the ligand-binding site and R508 co-ordinates to the backbone carbonyl N712 to form a salt bridge, removing this would

result in a more flexible loop. The residue 510 in HCS is also close to the putative carboxylase binding site, but this can not be reliably predicted from the E.coli BirA - BCCP structure due to very limited structural information and moreover, the human carboxylases are structurally not very similar to BCCP. Q699R occurs in a turn at the end of a β-strand and would not drastically change the packing of the molecule as it is in a solvent exposed loop. These observations suggest that these two variants do not majorly change the protein structure to affect its function. Thus probably the variants G510R and Q699R are presumed to be not critical for the protein folding or function. This inference agree with our kinetics data in this thesis: V_{max} values of variants G510R and Q699R were 73 and 101% of that of the wild type, and K_m values were 0.84 and 1.57 times that of the wild type, respectively. These kinetics data suggest that these two variants do not majorly change the catalytic activity of HCS, whereas V_{max} value of variant L216R was only 6% of the V_{max} in wild-type HCS and this variant causes multiple carboxylase deficiency (MCD) as mentioned above.

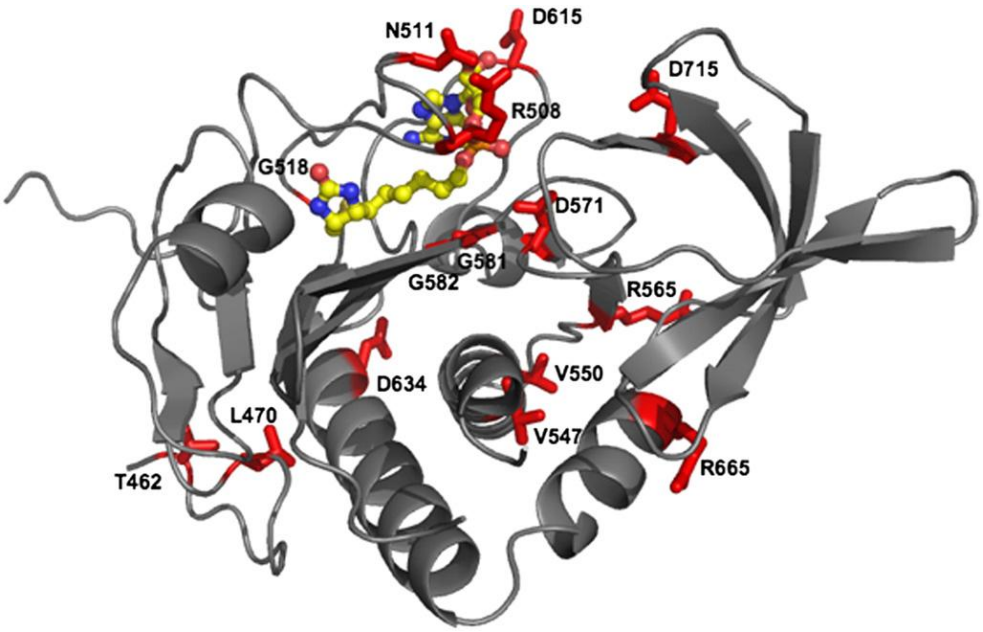


Fig. 12. R508W, N511K and other mutations in the central and C-terminal domains of HCS [Figure source: Pendini, Bailey et al. 2008]. Residues that are mutated in multiple carboxylase deficiency (MCD) are highlighted (in red) on the HCS model with biotinyl-5′-AMP shown in the active site (in stick and ball representation). It can be seen from this model that these mutations cluster around the biotin and ATP binding sites.

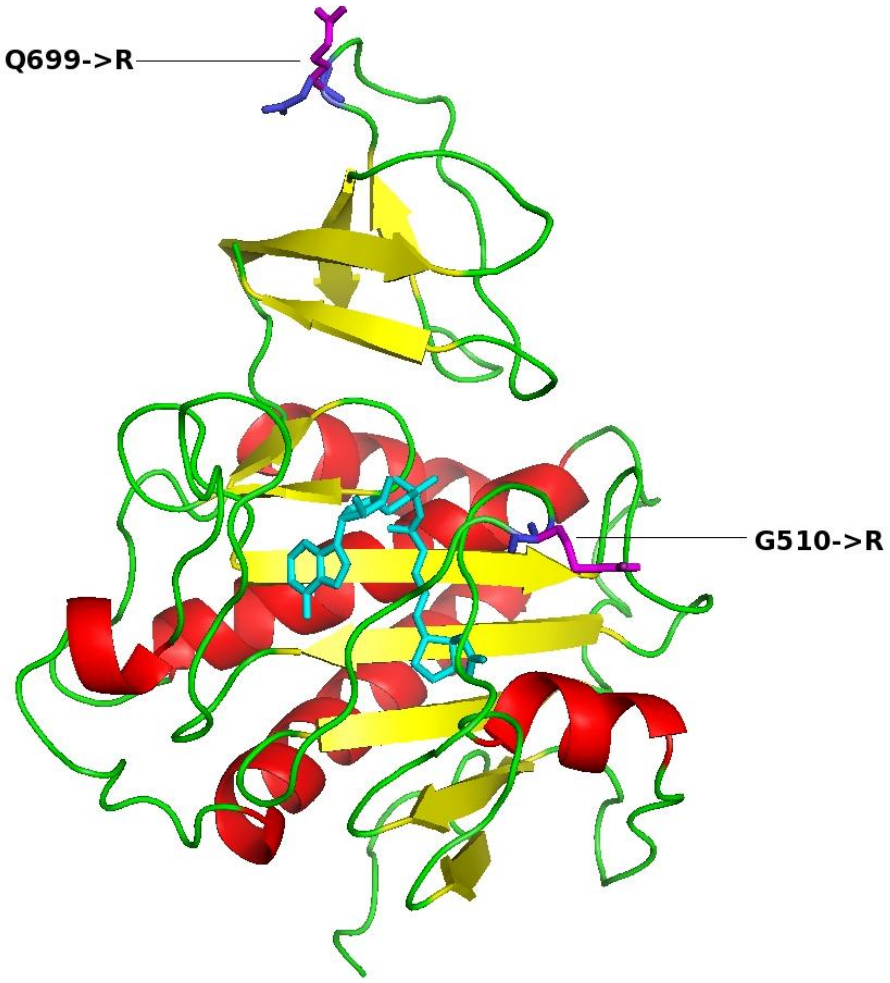


Fig. 13. Substitution sites 510 and 699 in the central and C-terminal domains of HCS. The substituted residues (510R and 699R) are highlighted in purple, while the wild type residues are highlighted in blue. The biotinyl 5'-AMP bound at the active site is highlighted in cyan. Substitution G510R occurs in one of the biotin-binding site (R508-S515 loop). Q699R occurs in a turn at the end of a β-strand. Either substitution site (510 and 699) is not involved in the biotin binding site and is located in the solvent exposed exterior of the protein.

In summary, V_{max} of variants L216R, V96F and G510R variant were 6%, 78% and 73%, respectively, of the V_{max} in wild-type HCS. The K_m values of the variants V96F and G510R were not significantly different from wild-type HCS. The activity of L216R was too low to allow for meaningful analysis of K_m. In contrast, the affinity of

variant Q699R for biotin was significantly lower than that of wild type HCS (K_m: 1.57 times that of wild type) and its V_{max} could be restored to that of wild-type HCS by biotin supplementation. This is the first biochemical characterization of catalytic activities of HCS variants. Also, this is the first report to show that HCS activity can be restored to normal by biotin supplementation.

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