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Substrate recognition characteristics of human holocarboxylase synthetase for biotin ligation

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ABSTRACT

Holocarboxylase synthetase (HCS) is an essential enzyme that catalyzes the incorporation of biotin into apo carboxylase and the biotinylation of the four biotin-dependent carboxylases in the human cell. Deficiency of HCS results in decreased activity of these carboxylases and affects various metabolic processes. Despite the importance of this enzyme, the recognition mechanism of the biotinoyl domain by human HCS (hHCS) has remained unclear. We have developed a method to express hHCS in the baculovirus system and used it to purify catalytically active, full-length hHCS. NMR experiments on the biotinoyl domains from acetyl-CoA carboxylase indicate that when hHCS is added, it recognizes the MKM motif in human and in *Escherichia coli* with a preference to the human biotinoyl domain. In addition, hHCS can biotinylate the biotinoyl domains from human and *E. coli* acetyl-CoA carboxylase at similar rates compared to the *E. coli* biotin protein ligase, BirA, which reacts very slowly with the human biotinoyl domain. We propose that the hHCS has greater substrate acceptability, while the BirA has higher substrate specificity. These results provide insights into substrate recognition by hHCS, which can be distinguished from BirA in this respect.

Introduction

Biotin is a water-soluble vitamin that serves as a cofactor for biotin-dependent enzymes that catalyze key metabolic reactions. In *Escherichia coli*, the only protein that is biotinylated is the biotin carboxyl carrier protein (BCCP) subunit of the acetyl-CoA carboxylase [1]. In contrast, four biotin-dependent enzymes [2] that play pivotal roles in human metabolism require biotin as a prosthetic group: acetyl-CoA carboxylase (ACC1 and ACC2), pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC). Both ACC1 and ACC2 catalyze the incorporation of bicarbonate into malonyl-CoA, a crucial regulatory step in fatty acid synthesis (ACC1) and mitochondrial fatty acid transport (ACC2). PC catalyzes the carboxylation of pyruvate to produce oxaloacetate in gluconeogenesis. MCC performs a single step in the breakdown of leucine to eventually yield acetyl-CoA and acetoacetate. Finally, PCC catalyzes the carboxylation of propionyl-CoA to methylmalonyl-CoA in the metabolism of odd-chain fatty acids [2].

Biotinylation of these carboxylases is essential for their enzymatic activities. The attachment of biotin to carboxylases via the N^6-amino group of a specific lysine residue is mediated by biotin protein ligase (BPL). Most organisms have only one enzyme for the attachment of biotin to the cognate proteins. This is known as the BirA protein in *E. coli* [3] and holocarboxylase synthetase (HCS) in eukaryotes [2,4]. For both BirA and HCS, the addition of biotin occurs in two-step manner. In the first step, BPL activates biotin at the expense of ATP to form the reaction intermediate biotinyl-5′-AMP, in which the carboxyl group of biotin is activated by the addition of an adenylate group. Subsequently, the biotin moiety of biotinyl-5′-AMP is transferred to the target protein [5,6]. This is a specific post-translational modification that is conserved in all life forms, as BPLs are interchangeable between organisms [7]. *E. coli* BirA can biotinylate apo-proteins from various sources: BCCP87 (*E. coli*) [8], p-86 (yeast) [9] and the human biotinoyl domain of ACC2 [10].

BPL recognizes a 60–90 amino acid domain that contains a specific lysine residue located within the sequence (Ala/Val)-Met-Lys-Met. All biotin-dependent enzymes share a high sequence homology within this sequence (Fig. 1A). This biotin-binding site is often part of a larger subunit of the carboxylase and is located within the biotinoyl domain [11]. The biotin prosthetic group is attached to a specific lysine residue in the biotinoyl domain to yield the holo form, which swings between the active sites of biotin carboxylase

Abbreviations: DSS, dimethylsilyl propionate; DTT, 1,4-dithiothreitol; HRP, horseradish peroxidase; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; CSP, chemical shift perturbation

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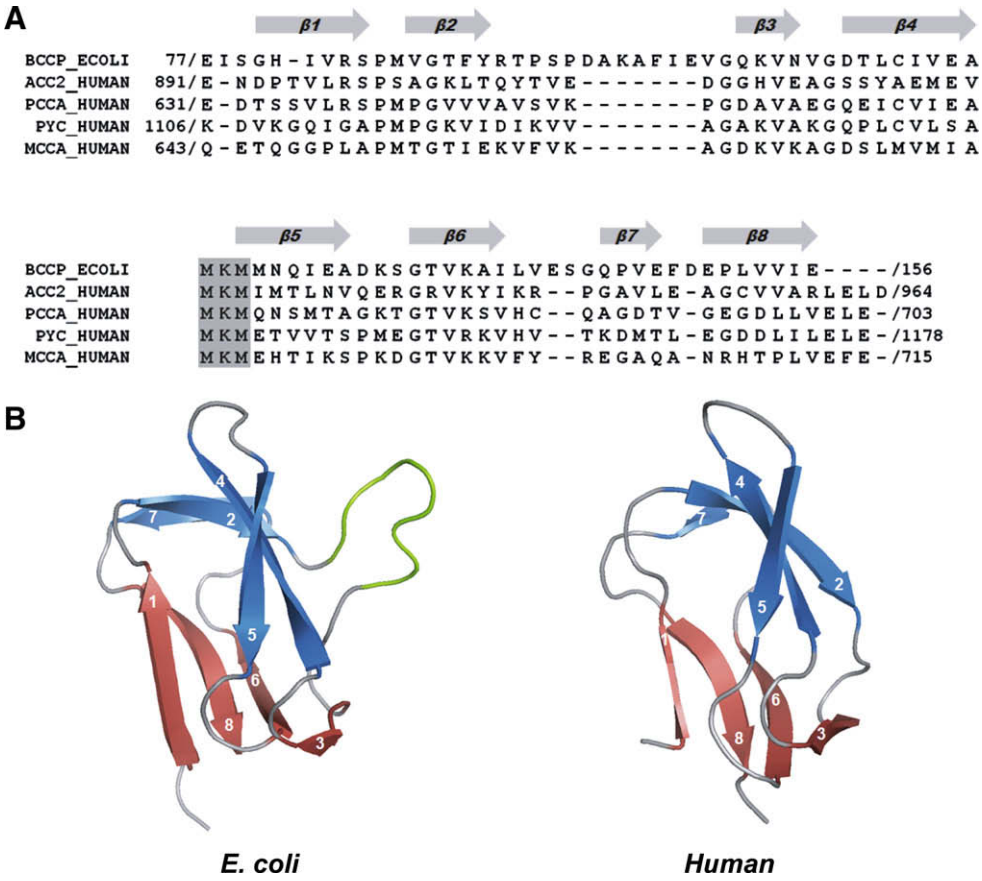

Fig. 1. Sequence alignment of the biotinoyl domains and the structural comparison between them. (A) The aligned sequences are denoted by their SwissProt identifiers (protein name_species), and the extent of the aligned region is indicated. The substrate proteins for biotinylation are aligned: BCCP, the biotin carboxyl carrier protein; ACC2, acetyl-CoA carboxylase2; PCCA, the α chain of propionyl-CoA carboxylase; PYC, pyruvate carboxylase; and MCCA, the α subunit of 3-methylcrotonyl-CoA carboxylase. The β-strands of the biotinoyl domain of *E. coli* BCCP are represented by arrows above the amino acid sequence and the MKM motif of each protein is shaded in grey. (B) The biotinoyl domains of *E. coli* BCCP (BCCP87) and human ACC2 (hACC75). The two β-sheets are red and blue, and the strands have been numbered consecutively. The specific lysine residue of each biotin acceptor is located in the hairpin between β4 and β5. *E. coli* thumb residues are shown in green.

(BC) and carboxyl transferase (CT) domain in carboxylase [12]. In the swinging arm model, biotin is positioned at the end of a long, flexible lysine side chain and this movement facilitates the translocation of CO_2 between BC and CT [13].

The structure of *E. coli* biotinoyl domain, as determined by X-ray crystallography [14] and NMR spectroscopy [15], is that of a flattened β-barrel, comprising two very similar four-stranded sheets with the biotinyl-lysine residue located in an exposed β-turn. More recently, a structural study of the biotinoyl domain of human ACC2 has been reported [10]. Although the structures have a similar folding topology, with a root mean square deviation of 3.31 Å for the backbone atoms, the human and *E. coli* biotinoyl domains show notable differences. First, the thumb structure of the *E. coli* biotinoyl domain [16], which is composed of eight protruding residues and interacts with the biotin moiety, is absent in the human biotinoyl domain (Fig. 1B). Second, the local structure in the consensus 'Met-Lys-Met' (MKM) motif is different between the two. In *E. coli*, the MKM motif is a type I' hairpin structure [17] and the geometry of its side chain is represented in a clockwise orientation. In contrast, the human ACC2 MKM motif is represented as a type I hairpin and the geometry of its side chain is counter-clockwise [10].

There are many studies on the recognition of MKM motifs by the *E. coli* biotin protein ligase, BirA, but the recognition process of the biotinoyl domain by human HCS (hHCS) has remained unclear. Recent studies about the functional characterization of hHCS demonstrate that N- and C-termini of hHCS are involved in the biotin acceptor substrate recognition [11,18,19]. However, these results did not provide information about how this hHCS recognizes the acceptor substrates structurally. Here we investigated the structural characteristics of the biotinoyl domain recognized by hHCS, which is important to elucidate the substrate recognition mechanism. To enable the study of hHCS, we have developed a method to express active hHCS using the baculovirus system. We used NMR spectroscopy to identify residues in the human ACC2 biotinoyl domain that are in contact with hHCS, and we have compared the biotinylation rate of human and *E. coli* biotinoyl domains using avidin blots. In comparison with the results from BirA, these results indicate that hHCS recognizes its substrate through a common interaction with the MKM motif, but has a higher acceptability for the different substrates.

Materials and methods

Vector construction. The hHCS gene was generated from a full-length cDNA clone encoding human HCS (726 aa), the kind gift of Dr. Yoichi Suzuki, in pBluescript. The PCR product was digested with EcoRI and NotI and inserted into a modified version of the pVL1393 baculovirus vector (BD Biosciences). The coding region of the baculovirus expression construct terminated with a 6× His-tag, followed by a TEV cleavage site that was fused to the C-terminus of hHCS.

Expression and purification of recombinant human HCS. The recombinant baculovirus for hHCS was generated and amplified in SF9 insect cells (Invitrogen). The resulting high-titer virus was used

to infect Hi5 insect cells (Invitrogen) growing in SF900 II SFM Medium (Gibco) at 28 °C. The incubation continued until the cells were harvested, 50-h post-infection. Hi5 cells infected with recombinant baculovirus were harvested by centrifugation and resuspended in 20 mM Tris–HCl (pH 8.0) containing 300 mM NaCl, 5 mM imidazole, 10% glycerol, 4 mM β-mercaptoethanol and protease inhibitor cocktail tablets (Roche). The cells were lysed using a constant cell disruption system. The cell lysate was centrifuged at 45,000*g* for 30 min at 4 °C and the supernatant was filtered through a 0.8 μm membrane. The clarified lysate was incubated with a nickel affinity resin for 30 min at 4 °C. After washing with 20 mM Tris–HCl (pH 8.0) containing 200 mM NaCl, 20 mM imidazole, 5% glycerol and 4 mM β-mercaptoethanol, the proteins that were bound to the column were eluted using 500 mM imidazole added to the same washing buffer. The eluate was diluted 5-fold with equilibration buffer (20 mM Tris–HCl (pH 8.0), 5% glycerol and 1 mM DTT) and applied to a Q-Sepharose column that had been equilibrated with the same buffer. The protein was eluted with a linear gradient of 0–0.5 M NaCl in the equilibration buffer. The peak fractions were pooled, concentrated in an Amicon Ultra device and loaded onto a Superdex 200 gel filtration column that had been equilibrated with 20 mM Tris (pH 8.0), 150 mM NaCl, 5% glycerol and 2 mM DTT. The fractions that contained hHCS were pooled and stored at −80 °C, and the purity of the protein sample was confirmed using SDS–PAGE under reducing conditions.

Expression and purification of BCCPs and BirA. The construction of a vector for the expression of the human ACC2 biotinoyl domain (denoted as hACC75 hereafter) has been previously described [10]. ^{15}N-labeled apo-protein was prepared from *E. coli* BL21(λDE3) containing the plasmid pET(hACC75-His_6), which encodes the 75-residue biotinoyl domain (residues 891–965 encoded by human *acc*2). The biotinoyl domain was ^{15}N-labeled by growing the cells in M9 medium in which $^{15}NH_4Cl$ was the sole source of nitrogen. The protein was purified as described previously [10]. The DNA sequences for the *E. coli* biotinoyl domain (pTM53) [20] and *E. coli* BirA (pHBA) [21] were provided by Dr. John E. Cronan, Jr. and Dr. Anne Chapman-Smith. The expression plasmid was created by cloning a BamHI–XhoI fragment of pTM53 into the pGEX-4T-1 vector (Promega) to increase the expression level. Briefly, *E. coli* BL21(λDE3) cells that had been transformed with the pGEX-4T-1-BCCP87 plasmid were cultured and treated with IPTG to induce protein production. The GST-fused protein was purified using a GST column (GSTrap FF 5, Amersham Bioscience) and purified further using a gel filtration column (Superdex 75, Pharmacia) after GST cleavage [10]. *E. coli* BirA was overexpressed and purified using essentially the same method described previously [20].

NMR spectroscopy. The activity of the samples was confirmed using NMR. This was performed at 30 °C on either 80 μM ^{15}N-labeled apo-hACC75 or ^{15}N-labeled apo-BCCP87, both containing 50 mM HEPES (pH 7.3), 3 mM ATP, 5.5 mM $MgCl_2$, 1 mM DTT, 500 μM biotin and 150 nM hHCS. For the chemical shift perturbation experiments, NMR spectra were recorded at 20 °C using samples that contained 1 mM ^{15}N-labeled apo-hACC75 in 50 mM HEPES (pH 7.3), 1 mM DTT, 10% D_2O and 20 μM dimethylsilyl propionate (DSS). The combined amide CSP was calculated as $\Delta\delta = [(\Delta\delta_N/5)^2 + (\Delta\delta_H)^2]^{1/2}$, where $\Delta\delta_N$ and $\Delta\delta_H$ are the shifts in the ^{15}N and ^{1}H signals, respectively [22]. All NMR data were recorded on Avance 500 spectrometers (Bruker), processed with NMRPipe and analyzed using Sparky 3.114.

In vitro biotinylation assays. The biotinylation of the apo human and *E. coli* biotinoyl domains was detected quantitatively using avidin blots. The reactions were carried out in the mixtures described above (50 mM HEPES (pH 7.3), 3 mM ATP, 5.5 mM $MgCl_2$, 1 mM DTT and 500 μM biotin) and initiated by the addition of purified hHCS or BirA to a final concentration of 15 nM at 30 °C. Samples of the reaction mixture that were taken at constant time intervals were resolved using an 18% polyacrylamide gel and transferred to a PVDF membrane. The non-specific sites on the membrane were blocked using 1% BSA and then incubated with streptavidin-HRP (1:1000) for 1 h at room temperature. The membrane was developed using AEC/H_2O_2. The biotinylated proteins were analyzed using ChemiDoc XRS (Bio-Rad) and Quantity One software. The extent of the biotinylation of the human and *E. coli* biotinoyl domains by hHCS was further confirmed using NMR.

Results

Protein expression and purification

We have successfully expressed and purified recombinant, full-length, human HCS (hHCS) with a C-terminal hexahistidine tag from a Hi5 insect cell culture. However, in addition to the 83 kDa protein band that corresponds to full-length His_6-hHCS, we observed a 65 kDa band on the SDS–PAGE gel of the pooled fractions from the Q-Sepharose column (Fig. 2 lane 3). This band was also detected on a Western blot using a His-tag antibody, indicating that the C-terminal His_6 tag was present, and therefore that the N-terminal part had been degraded (data not shown). N-terminal sequencing of the 65 kDa band indicated that 117 residues were cleaved from the N-terminus. The full-length, intact hHCS was purified further by gel filtration chromatography, resulting in almost pure, full-length hHCS as determined using SDS–PAGE (Fig. 2 lane 4). The activity of the purified protein was confirmed by NMR spectroscopy and the avidin blot assay as described in the following section.

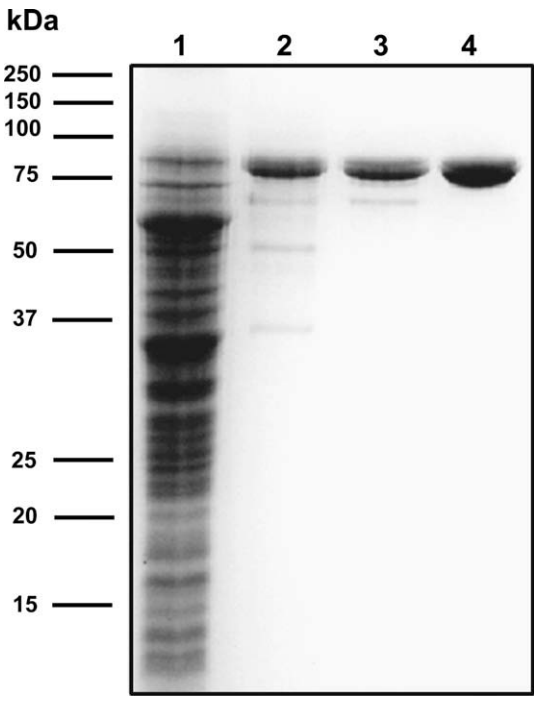

Fig. 2. Purification of human HCS from Hi5 cells that expressed the full construct. Samples from all purification steps were resolved by SDS–PAGE on a 12% gel. A total of 5 μg protein was loaded in each lane. The hHCS band is indicated. Lane 1, the soluble fraction of hHCS lysate; lane 2, the Ni resin pooled eluates; lane 3, the Q Sepharose pooled eluates; lane 4, the Superdex 200 eluate (the final purified protein). The migration of the molecular mass markers is indicated on the left.

Confirmation of the activity of hHCS by NMR

NMR spectral changes are observed for residues near the MKM motif when the ^{15}N-labeled biotinoyl domain is biotinylated by biotin protein ligase [10]. To investigate the activity of purified hHCS, we monitored the biotinylation of the human and *E. coli* biotinoyl domains using ^{1}H–^{15}N HSQC spectra. After 12 h, HSQC spectrum of the human ACC2 biotinoyl domain (Glu 891-Asn 965, hACC75) showed small but significant chemical shift changes that were localized to residues around Lys 929 (i.e., Glu 926, Val 927, Met 928, Met 930 and Ile 931), where the biotin cofactor is covalently attached (Supplementary Fig. S1A).

Biotinylation of the *E. coli* biotinoyl domain (BCCP87), on the other hand, showed significant chemical shift changes, not only for residues that near the biotinylated lysine (Lys 112), but also for residues in the thumb region (from Gly 89 to Phe 102) due to direct interactions with the biotin group (Supplementary Fig. S1B). These spectral changes that occur when hHCS is added to the biotinoyl domains indicate that the hHCS that we purified was active and that *in vitro* biotinylation with hHCS was successful.

Binding study of hHCS using NMR

We performed chemical shift perturbation (CSP) experiments of *E. coli* BCCP87 and human ACC75 in the presence of hHCS to understand the characteristics of substrate recognition by this enzyme. ^{1}H–^{15}N HSQC spectra of apo-hACC75 and BCCP87 were recorded in the presence and absence of the hHCS enzyme. The chemical shift changes experienced by residues in the biotinoyl domain are summarized in Fig. 3.

When hHCS was added to hACC75 in solution, conspicuous chemical shift changes in the backbone amide signals of hACC75 were observed for the residues from Val 927 to Met 930. These residues correspond to the conserved (Ala/Val) MKM sequence (Fig. 3A). The largest chemical shift change was 0.020 ppm for Lys 929, the specific lysine residue where the biotin attaches. Chemical shift changes in the HSQC spectrum indicate changes in the chemical environment of the residues due to protein–protein interactions. Thus, this result demonstrates that the conserved MKM motif of hACC75 plays a role in the binding with hHCS. As a control, the same experiment was repeated in the presence of 0.45 mM BSA instead of hHCS. BSA showed no specific binding (Fig. 3B).

The binding pattern for BCCP87 with hHCS was essentially the same as that of hACC75. The largest chemical shift change was 0.026 ppm for Lys 122, the biotin-binding lysine. The conserved MKM motif of BCCP87 showed remarkable changes on binding to hHCS (Fig. 3C) as compared to the control experiment with BSA (Fig. 3D).

These results indicate that hHCS recognizes the MKM motif. This recognition is characteristic of the biotinoyl domain and occurred in both *E. coli* BCCP87 and human ACC75. Compared to the bacterial biotin protein ligase BirA, which recognizes the additional residues from the "thumb" region of BCCP87 [23], hHCS does not interact with the "thumb" region and recognizes a smaller area.

Monitoring of biotinylation by avidin blotting

The biotinylation of *E. coli* BCCP87 and human ACC75 was monitored by avidin blotting using streptavidin conjugated to horse-

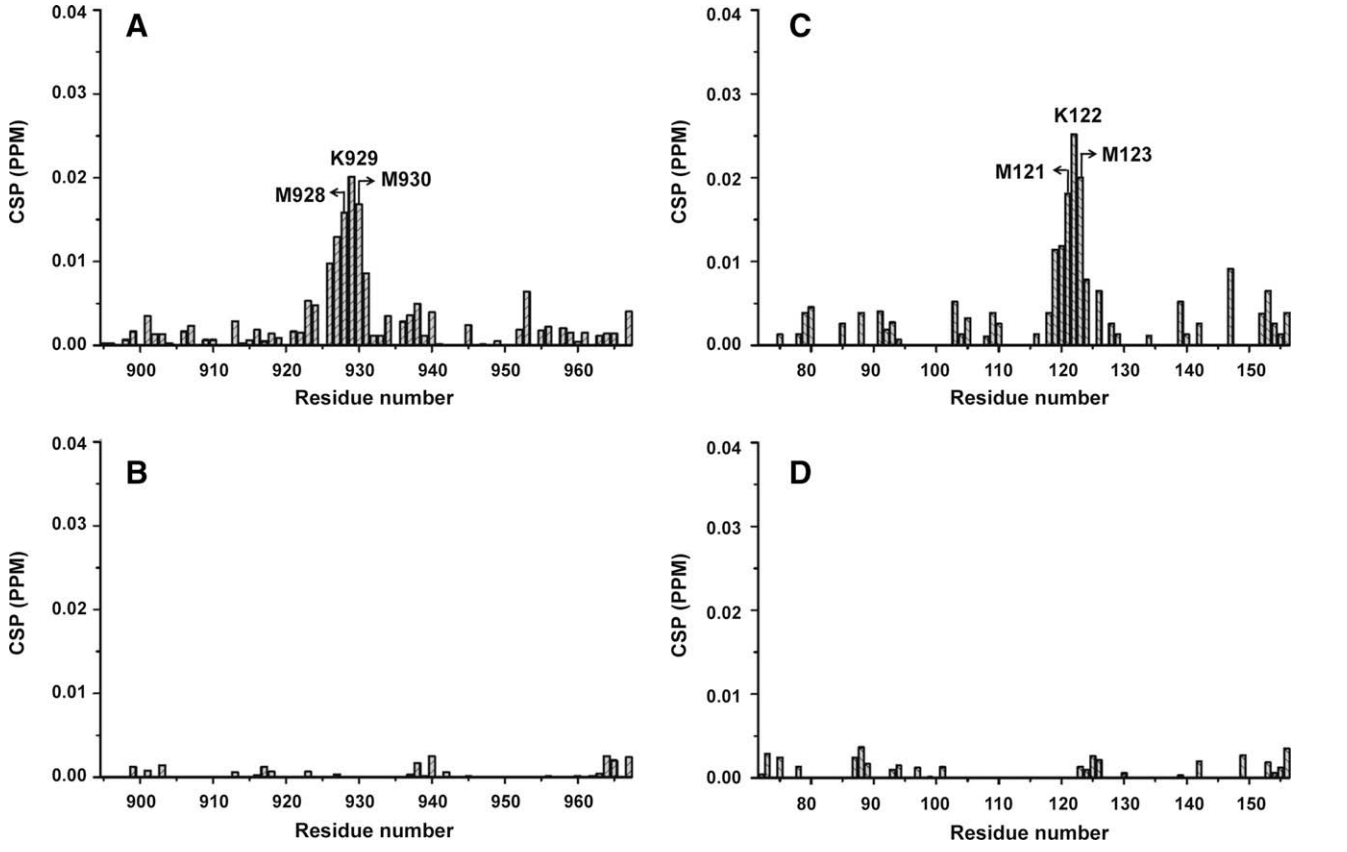

Fig. 3. The analysis of the chemical shift changes of the human biotinoyl domain. (A) The plot represents the combined amide CSP differences between the HSQC spectra of apo-hACC75 in the presence and absence of hHCS. The result for control BSA mixed with apo-hACC75 is plotted in (B). The CSP differences between apo BCCP87 in the presence and absence of hHCS are plotted in (C), and the corresponding data for the control, BSA, are plotted in (D). The CSP is plotted against the residue number for each domain, and the residues that have significantly shifted resonances are indicated.

radish peroxidase (HRP) (Fig. 4). To observe their relative activities in detail, the lower concentrations (15 nM) of both enzymes were used for avidin blot than for activity confirmation by NMR (150 nM, see Materials and methods).

hACC75 and BCCP87 were biotinylated by human HCS, yielding saturation bands on an avidin blot at 5 and 12 h after initiation of the reactions, respectively. On the other hand, with *E. coli* BirA, only BCCP87 showed a saturation band at 5 h after the initiation, and the other biotinoyl domain, hACC75, did not reach saturation until 12 h. These results indicate that hHCS can biotinylate different substrates within a similar range of rates, while BirA reacts very slowly with hACC75. We suggest that hHCS has greater substrate acceptability and that BirA has higher substrate specificity.

Comparing the times taken to reach saturation for the two substrates of hHCS, the biotinylation of hACC75 was faster than that of BCCP87. Considering that hHCS recognizes similar surfaces on the substrate, including MKM motifs, it is clear that hHCS recognizes the conformation of the MKM motif of human ACC75 preferentially, although it is susceptible to all the substrates mentioned above.

Discussion

This study presents the biophysical and functional aspects of the recognition of the biotinoyl domain by hHCS that result in biotinylation of the human and *E. coli* biotinoyl domains. This is the critical step for many metabolic processes in the cell. Despite much investigation of the recognition of MKM motifs by *E. coli* BirA, few aspects of the recognition of the biotinoyl domain by human HCS have been reported. Studies were limited due to the difficulties to obtain the enough amounts of the recombinant full-length hHCS for the experiments. The hHCS protein has been also purified from placenta [24] and expressed in *E. coli* in an N- and C-terminal-deleted form [11]. During submission of our manuscript, the full-length hHCS expression in *E. coli* and their activities by kinetic analysis were reported [19]. On the other hand, we have developed a protocol to produce active recombinant hHCS using an insect cell system. As a result, our baculovirus system produces ten times larger amount than the reported yield from *E. coli* expression.

Here we show that hHCS can bind to hACC75 and to BCCP87 through interaction with the MKM motif in two biotinoyl domains. In our previous study, we performed CSP experiments at the low concentration of *E. coli* BirA, which caused the intermediate changes in chemical shifts and line broadening for the affected cross peak [10]. For direct comparison between our result of hHCS and that from Reche et al., the study of substrate recognition by BirA, we performed CSP at the concentration suggested by Reche et al. [23] in this study.

We demonstrated that the biotinylation of *E. coli* and human biotinoyl domains by BirA (150 nM) using NMR spectroscopy and avidin blot in the prior study. When the final concentration of 150 nM BirA was used for reaction, it took 30 min and 5–6 h to saturate biotinylation of *E. coli* and human biotinoyl domain, respectively. Thus, we suggested different structural features of biotinoyl domains between *E. coli* and human [10]. These results are consistent with this study (15 nM of BirA) in that biotinylation rate of

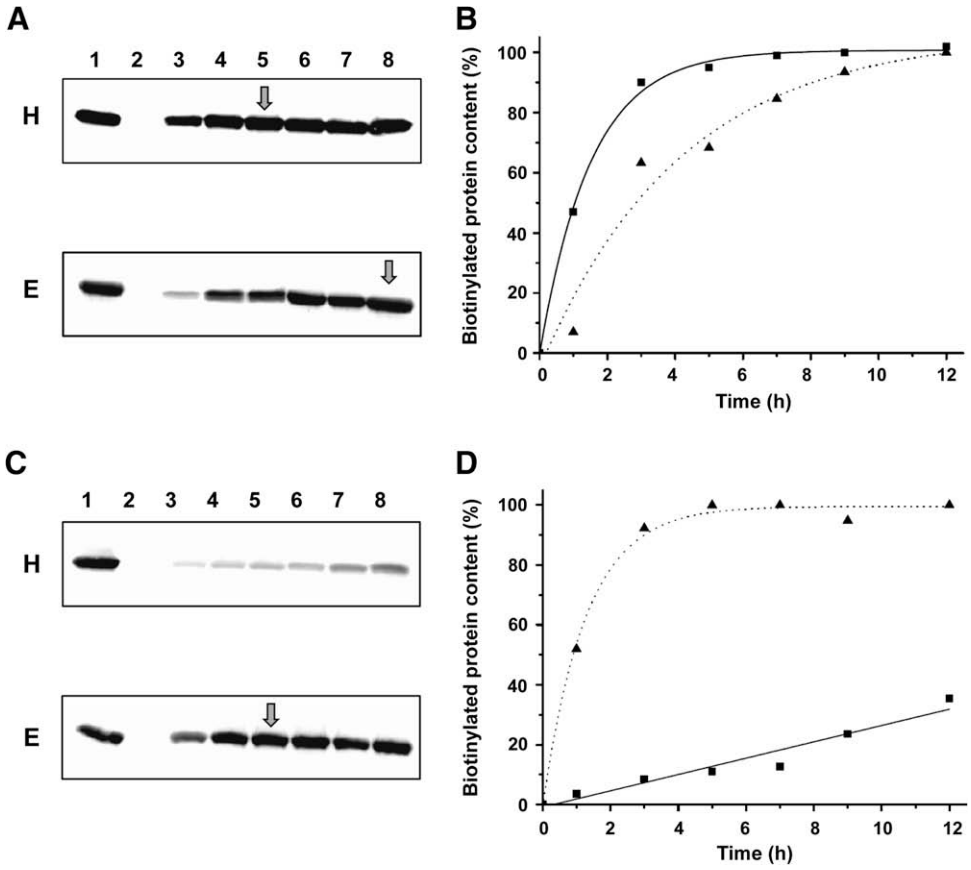

Fig. 4. Avidin blots of the holo-biotinoyl domains of human and *E. coli*. The bands show the biotinylation pattern of human (H) and *E. coli* (E) proteins at 30 °C. These were taken at 2 h time increments following the addition of hHCS (A) and BirA (C). Lane 1, identical amounts of each holo protein as a control; lane 2, no ligase added (before biotinylation); lanes 3–8 correspond to 1, 3, 5, 7, 9 and 12 h after the addition of the ligase, respectively. The amounts of biotinylated protein during the time courses are also plotted in (B) and (D), using hHCS and BirA, respectively. The symbols ■ with the solid line and ▲ with the dotted line indicate the band intensities of human ACC75 and *E. coli* BCCP87, respectively.

BirA shows significant difference in *E. coli* and human biotinoyl domain.

Interestingly, hHCS can react with different substrates with similar biotinylation rates, demonstrating high substrate acceptability. BirA does not possess this attribute, possibly due to the "thumb" recognition. Indeed, hHCS can recognize four different endogenous substrates, i.e., ACC, PCC, PC and MCC [25]. Thus, we propose that hHCS requires a certain degree of substrate acceptability to accomplish its function in the various metabolic processes of the cell.

We have demonstrated conformational differences between MKM motifs from hACC75 and BCCP87 [10] that can be recognized by BirA. These differences have also been demonstrated here. From these results, we suggest that hHCS can distinguish between alternative local geometries of the MKM motif. However, substrate recognition by *E. coli* BirA has been attributed to the "thumb" region in addition to the MKM motif [10,21], and it leads BirA to have higher substrate specificity than hHCS. Further structural studies to elucidate the determinants for recognition of the MKM motif are needed.

In conclusion, we have demonstrated biotinylation activity using our recombinant human HCS. We also measured changes in the biotinylation rates that result from preferential substrate recognition by these enzymes. These results provide structural insights into the biotinylation that occurs during various metabolic processes such as gluconeogenesis, amino acid catabolism and fatty acid metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.079.

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