

# **A unique biotin carboxyl carrier protein in archaeon *Sulfolobus tokodaii***

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## **Abstract**

Biotin carboxyl carrier protein (BCCP) is one subunit or domain of biotin-dependent enzymes. BCCP becomes an active substrate for carboxylation and carboxyl transfer, after biotinylation of its canonical lysine residue by biotin protein ligase (BPL). BCCP carries a characteristic local sequence surrounding the canonical lysine residue, typically -M-K-M-. Archaeon *Sulfolobus tokodaii* is unique in that its BCCP has serine replaced for the methionine C-terminal to the lysine. This BCCP is biotinylated by its own BPL, but not by *Escherichia coli* BPL. Likewise, *E. coli* BCCP is not biotinylated by *S. tokodaii* BPL, indicating that the substrate specificity is different between the two organisms.

*Keywords:* biotin carboxyl carrier protein; biotin protein ligase; biotinylation, substrate specificity; *Sulfolobus tokodaii*

*Abbreviations:* BCCP, biotin carboxyl carrier protein; BPL, biotin protein ligase

## 1. Introduction

Biotin-dependent enzymes concern carboxylation of such key metabolites as acetyl-CoA and pyruvate, carboxyl transfer and decarboxylation [1,2]. They are composed of some or all of the following domains or subunits, biotin carboxylase (BC), carboxyl transferase (CT) and biotin carboxyl carrier protein (BCCP). The former two serve as enzymes, while the latter as substrate for them. BCCP becomes active only after biotinylation of its canonical lysine residue by biotin protein ligase (BPL) or holo carboxylase synthetase (HCS) [3-5]. The canonical lysine (K) of BCCP is flanked by hydrophobic amino acids, typically in the form of -M-K-M- in most cases but either one or both of the methionines (M) may be substituted by leucine or valine. Likewise, BPL from various organisms seems to share common structural and catalytic features [4-6] and in fact a BPL from one organism can usually biotinylate the BCCP domain from other organisms [4,5,7,8]. Archaeon *Sulfolobus tokodaii* is unique in that the residue C-terminal to the canonical lysine is substituted with serine (S), a residue significantly different in size and nature from methionine or related amino acids. During the course of our studies on biotinylation of this BCCP by BPL from *Escherichia coli*, a marked difference in the substrate specificity was observed from that of *E. coli*. This unprecedented finding is herein explored further with mutants of BCCP, where the serine of *S. tokodaii* BCCP was converted into methionine and the methionine of *E. coli* BCCP was converted into serine.

## 2. Materials and methods

### 2.1. Materials

Oligonucleotides used as PCR primers were custom synthesized by Hokkaido Science (Sapporo, Japan) and enzymes used for gene manipulation were purchased from Takara (Kyoto, Japan). Vectors pET21a (ampicillin resistant) and pET24a (kanamycin resistant) were from Novagen (Madison, WI) and the TOPO TA cloning kit from Invitrogen (Carlsbad, CA). The QIAquick gel extraction kit was obtained from Qiagen (Valencia, CA) and the CEQ<sup>TM</sup>DTCS quick start kit, Beckman Coulter (Fullerton, CA). Diethylaminoethyl

(DEAE) cellulose and 3MM filter paper were the products of Whatman (Maidstone, UK). Butyl Toyopearl was purchased from Tosoh (Tokyo, Japan) and HiLoad 26/60 Superdex 200 pg from Amersham Biosciences (Piscataway, NJ). [<sup>3</sup>H]Biotin (specific activity 39.0 Ci/mmol) was obtained from Amersham. Other common chemicals were obtained from local suppliers.

## 2.2. Apparatus

Nucleotide and amino acid sequences were determined on a CEQ8000 sequencer from Beckman and ABI491 sequencer from Perkin-Elmer (Foster City, CA), respectively. Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectra were determined on a Voyager DE-STR mass spectrometer (PerSeptive Biosystems, Framingham, MA). Radioactivity was measured on an LS6500 liquid scintillation counter from Beckman.

## 2.3. Construction of over-expression plasmids

Over-expression plasmids for *S. tokodaii* BCCP and BPL were prepared as follows. It is noted that the 51 bases from the putative initiating methionine of the BCCP gene and the putative first codon, GTG, of the BPL gene were omitted. Thus, the truncated gene for BCCP (456 bp) and the BPL gene (696 bp) were amplified by polymerase chain reaction with shot gun clone STLGR14596 or STLGR11133 for *S. tokodaii* as template [9] and the oligonucleotides listed in Table 1 as primers (e. g., combination of F1 and B1) under the following conditions: after heating at 94 °C for 5 min, the following cycle was repeated 30 times; 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min, and finally heated at 72 °C for 6 min. The PCR products were TA cloned into TOPO vector, sequenced and re-cloned into pET21a or pET24a for BCCP and BPL, respectively, to give pstBCCP and pstBPL. Over-expression plasmids for *E. coli* BCCP and BPL, pecBCCP and pecBPL, respectively, were prepared analogously [10,11].

The mutants pstBCCPm and pecBCCPm were prepared by two-step PCR. Thus, upstream and downstream regions relative to the site of mutation (Ser-136 in *S. tokodaii* and Met-123 in *E. coli*) were amplified separately with pstBCCP or pecBCCP as template and the

following combinations of primers, F1 and B2, F2 and P-pET21a representing part of the vector sequence. The two fragments each thus obtained were recovered and allowed to anneal and extend. The second PCR was run with a combination of F1 and B1, and the product was sequenced and re-cloned into pET21a.

#### 2.4. Protein purification

*S. tokodaii* BCCP was purified as follows. *E. coli* BL21(DE3) harboring pstBCCP or pstBCCPm was grown in 1 L of Luria-Bertani (LB) broth supplemented with 50 µg/mL ampicillin and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) in 10 mL batches, as the level of over-expression was suppressed drastically on a larger scale. Cells collected by centrifugation were disrupted by sonication. Following centrifugation, the supernatant was subjected successively to ammonium sulfate fractionation, DEAE cellulose and butyl Toyopearl chromatography to yield nearly homogeneous protein. Typically, 20 and 50 mg of wild type and mutated BCCP were obtained, respectively. No significant difference was observed in the behavior of the wild type and mutant BCCP.

*S. tokodaii* BPL was purified analogously from a transformant of *E. coli* BL21(DE3) harboring pstBPL, grown in the presence of 50 µg/ml kanamycin and 1 mM IPTG. The harvested cells were disrupted by sonication, and following centrifugation, the protein was purified from the supernatant by DEAE cellulose, butyl Toyopearl, and finally gel filtration chromatography on HiLoad 26/60 Superdex 200 pg. Typically, 50 mg of *S. tokodaii* BPL was obtained from a 1 L culture.

*E. coli* BCCP and BPL were purified in a way analogous to that for the *S. tokodaii* counterparts except that the former was finally subjected to avidin affinity chromatography to separate the apo form from the holo form [12,13].

#### 2.5. Steady state kinetic analysis of biotinylation

The activity of *E. coli* and *S. tokodaii* BPL was determined by measuring the incorporation of [<sup>3</sup>H]biotin into either wild type or the mutated apo BCCP as described by Chapman-Smith et al. [14]. Briefly, unless otherwise stated, the assays contained 50 mM

Tris-HCl, pH 8.0, 3 mM ATP (*E. coli* BPL), 0.5 mM ATP (*S. tokodaii* BPL), 5.5 mM MgCl<sub>2</sub>, 5 μM biotin, 50 nM [<sup>3</sup>H]biotin (specific activity 39.0 Ci/mmol), 100 mM KCl, 0.1 mM dithiothreitol (DTT) and 0.1 mg/ml bovine serum albumin. The BCCP concentration adopted was as follows: 0.2 – 10 μM *S. tokodaii* BCCP with *S. tokodaii* BPL; 2 – 50 μM *E. coli* BCCP with *E. coli* BPL; 2 - 100 μM mutated BCCP from *S. tokodaii* or *E. coli* with *S. tokodaii* or *E. coli* BPL. The reaction was initiated by addition of purified *S. tokodaii* or *E. coli* BPL to a final concentration of 0.01-1 μM and incubated at 37 or 70 °C for up to 30 min. Aliquots taken at various time intervals were spotted onto dry 2 x 2 cm squares of Whatman 3MM paper, to which 100 μl of 5 mM biotin and 100 μl of 10% trichloroacetic acid had previously been placed. After air-drying, the filters were washed batchwise twice in ice-cold 10% trichloroacetic acid and once in ethanol, dried, and the acid-insoluble radioactivity measured.

### 3. Results

#### 3.1. Expression and purification of BCCP and BPL

There seems to be only one biotin-dependent enzyme in *S. tokodaii* as deduced from sequence homology with known eukaryotic, eubacterial and archaeal counterparts. This enzyme is presumably multi-functional acyl-CoA carboxylase and encoded by genes ST0593, ST0592 and ST0591, which are supposed to be its BC, BCCP and CT subunits, respectively [9,15,16]. In addition to them, there is BPL, which converts apo BCCP into active holo BCCP by biotinylation of its canonical lysine residue. BPL seems to be encoded by ST1525 [9] and this notion was verified by the fact that the putative BPL biotinylates apo BCCP as described below.

Both of *S. tokodaii* BCCP and BPL were over-expressed in *E. coli* and purified to homogeneity by conventional methods (Fig. 1, lanes 1 and 5). Purified proteins were analyzed by means of MALDI TOF mass spectrometry and N-terminal sequencing. Thus, the N-terminal sequences up to the fifth cycle from the second residue, TYDQQ for BCCP and (C)ISML for BPL, were consistent with the theoretical ones. In addition, the mass

observed for BCCP and BPL was  $16\,797 \pm 10$  and  $26\,633 \pm 32$ , respectively (mean  $\pm$  SD from four determinations), values consistent with theoretical masses of 16 798 and 26 620 within experimental error. It was inferred also by gel-filtration chromatography that *S. tokodaii* BCCP and BPL exist as a monomer each (data not shown), a phenomenon in contrast to the *E. coli* counterparts, which assume oligomeric or dimeric states, respectively [17,18]. It is noted that *S. tokodaii* BCCP was not biotinylated at all even where *E. coli* cells were grown in medium containing 1  $\mu$ M biotin. This was further proven by avidin blot as described below.

Analogously, *E. coli* BCCP and BPL were over-expressed separately in *E. coli* and purified to homogeneity (Fig. 1, lanes 3 and 6). As this BCCP is biotinylated by endogenous BPL partially, purified BCCP was finally subjected to avidin affinity chromatography to separate the apo form from the holo form [12,13]. The mass of  $16\,689 \pm 10$  Da for this protein was consistent with the expected value of 16 687 Da. Likewise, the mass of  $35\,314 \pm 32$  Da for *E. coli* BPL was consistent with the theoretical value of 35 312 Da.

### **3.2. Substrate specificity of *S. tokodaii* biotinylation**

As described above, it was suggested that *S. tokodaii* BCCP is not biotinylated by *E. coli* BPL and that the substrate specificity of BPL may be more stringent than that perceived previously [5,7,8,19]. To assess this phenomenon quantitatively, in vitro biotinylation of BCCP by BPL was studied in various combinations of substrate and enzyme. Thus, 20  $\mu$ M BCCP and 2 or 20  $\mu$ M BPL were incubated at pH 8.0 at 37 °C for 2 h and the mixture was analyzed by avidin blot and mass spectrometry. The reaction was virtually complete under these conditions in the proper combinations; the peak for apo BCCP was shifted to a higher mass corresponding to that for holo BCCP:  $17\,026 \pm 6$  Da (17 024 in theory) for *S. tokodaii* and  $16\,917 \pm 12$  Da (16 913 in theory) for *E. coli*. It was found that *S. tokodaii* BCCP was not biotinylated by *E. coli* BPL, nor was *E. coli* BCCP by *S. tokodaii* BPL (Fig. 2, lanes 2 and 3). It is hence evident that the substrate specificity of biotinylation is totally different between *E. coli* and *S. tokodaii*. To the authors' knowledge, this is the first observation of

such a phenomenon.

### 3.3. Origin of substrate specificity

To clarify the origin of substrate specificity observed above, amino acid sequences of BCCP from various sources were compared (Fig. 3). Although overall sequence homology is not high across species, limited similarity is observed for the C-terminal regions, especially around the canonical lysine residue in the form of -E-X-M-K-M-. Nevertheless, there is at least one notable difference between the sequences of *S. tokodaii* and other organisms; the canonical lysine is flanked by methionines in *E. coli* and many other organisms, though one or both of them are replaced with leucine or valine in some cases (not shown)[8]. In the case of *S. tokodaii* BCCP, the C-terminal methionine is replaced with serine, an amino acid markedly different from methionine or leucine in terms of size and nature of the side chain group. It was hence supposed that this substitution dictates the substrate specificity of BCCP. To test this hypothesis, two mutants of BCCP were prepared where the serine of *S. tokodaii* BCCP was replaced with methionine (S136M) and the methionine of *E. coli* BCCP replaced with serine (M123S). The two mutants were purified to homogeneity just like wild type BCCP (Fig. 1, lanes 2 and 4).

Substrate activity of these mutated as well as wild type BCCP was assessed by steady state kinetics and the parameters obtained are compiled in Table 2. It is noted that the biotinylation of *S. tokodaii* BCCP by *S. tokodaii* BPL alone was carried out at 70 °C, as the reaction does not turn over at 37 °C. Biotinylation of the S136M mutant of *S. tokodaii* BCCP by *S. tokodaii* BPL was suppressed significantly mainly because of its decreased affinity for the enzyme. By contrast, the M123S mutant of *E. coli* BCCP was biotinylated now, albeit weakly, by *S. tokodaii* BPL, but it was still a much poorer substrate than the S136M mutant of *S. tokodaii* BCCP in terms of  $k_{cat}/K_m$ .

Likewise, biotinylation of the M123S mutant by *E. coli* BPL was suppressed drastically from that for the wild type. Especially, the  $K_m$  of the mutant increased 10 times, implying that its affinity for BPL decreased markedly. On the other hand, the S136M mutant of *S. tokodaii* BCCP now served, albeit weakly, as a substrate for *E. coli* BPL. Yet, this mutant

was a much poorer substrate than the M123S mutant of *E. coli* BCCP for *E. coli* BPL.

Incidentally, the  $K_m$  of *S. tokodaii* BPL for ATP and biotin was not different much from those of *E. coli* BPL for these substrates [20]:  $0.47 \pm 0.10$  mM for ATP and  $0.10 \pm 0.01$   $\mu$ M for biotin;  $0.28 \pm 0.03$  mM for ATP and  $0.18 \pm 0.03$   $\mu$ M for biotin in *E. coli*. It is also noted that substrate inhibition was observed for ATP at its high concentrations (above ca 0.4 mM) in the biotinylation of the S136M mutant of *S. tokodaii* BCCP by *S. tokodaii* BPL and the data were analyzed by taking this into consideration,  $K_I$   $0.62 \pm 0.13$  mM [21].

#### 4. Discussion

As described above, the substrate specificity in biotinylation of BCCP is totally different between *S. tokodaii* and *E. coli*. It was postulated that the residue C-terminal to the canonical lysine of BCCP, serine or methionine, is responsible for the difference in substrate specificity. This notion was tested by converting the serine to methionine in *S. tokodaii* BCCP and the methionine to serine in *E. coli* BCCP. The resulting mutants, S136M and M123S, now served as substrate for the heterologous BPL, but the magnitude of their activity was much smaller than expected, indicating that this residue is not the sole determinant of the substrate specificity in biotinylation of BCCP; in fact, it was reported that substitution of the methionine residues flanking the canonical lysine does not affect biotinylation of the mutants significantly [22,23]. Although we envisage that the three-dimensional structure of *S. tokodaii* BCCP resembles that of the *E. coli* counterpart [24], the local structure, especially surrounding the canonical lysine, may be subtly different, so that BPL cannot recognize BCCP from the other source as a proper substrate. It is, however, only after the three-dimensional structure of *S. tokodaii* BCCP and BPL becomes available when detailed discussion on their interaction is possible.

As described above, the local sequence surrounding the canonical lysine of BCCP is conserved in many organisms including archaea in the form of -E-X-M-K-M-, but Crenarchaea or *Sulfolobaceae* alone carry less bulky serine (*S. tokodaii*, *S. acidocaldarius*, *S. metallicus*, *Acidianus brierleyi* and *Metallosphaera sedula*) or alanine (*S. solfataricus*) in place of the C-terminal methionine. The physiological significance of this substitution

remains to be clarified, however. Additionally, it is noted that there are five conserved glycine residues near the biotin-binding site of BPL from *E. coli* and *Pyrococcus horikoshii* [25,26]. Of interest is the fact that only one of them, Gly-115 in *E. coli* and Gly-45 in *P. horikoshii*, is mutated into more bulky alanine in *Sulfolobaceae* alone, Ala-43 in *S. tokodaii*. This substitution may compensate the mutation of the methionine into serine in BCCP in those organisms. Further investigation is clearly needed to test this hypothesis, and mutagenesis studies are under way in this laboratory.

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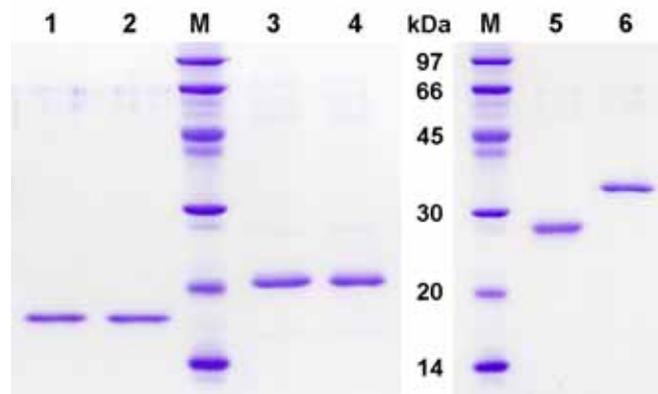
## Figure legends

Fig. 1. SDS-PAGE of purified BCCP and BPL with 13% polyacrylamide gel and 0.5 - 0.8  $\mu$ g of the protein: M, molecular size marker; lane 1, *S. tokodaii* wild-type BCCP; lane 2, *S. tokodaii* S136M BCCP; lane 3, *E. coli* wild-type BCCP; lane 4, *E. coli* M123S BCCP; lane 5, *S. tokodaii* BPL; lane 6, *E. coli* BPL.

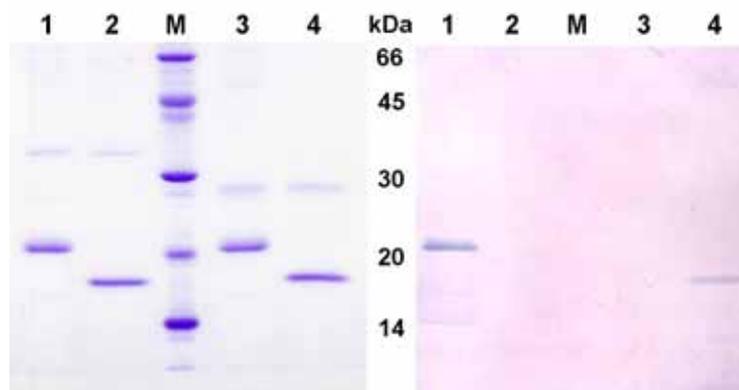
Fig. 2. Avidin blot analysis of *in vitro* biotinylation of 20  $\mu$ M BCCP and 2  $\mu$ M BPL at 37  $^{\circ}$ C for 2 h under the same conditions as those used for steady state kinetic analysis (see Section 2.5.): lane 1, reaction of *E. coli* BCCP with *E. coli* BPL; lane 2, *S. tokodaii* BCCP with *E. coli* BPL; lane 3, *E. coli* BCCP with *S. tokodaii* BPL; lane 4, *S. tokodaii* BCCP with *S. tokodaii* BPL. Aliquots from each reaction solution was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (A). Following SDS-PAGE, the proteins were electroblotted on a membrane and detected by the reaction with alkaline phosphatase-conjugated avidin (B) [12].

Fig. 3. Alignment of partial amino acid sequences of BCCP around the canonical lysine residue from *S. tokodaii* (Sto) acyl-CoA carboxylase (NCBI protein sequence database accession number ST0592), *E. coli* (Eco) acetyl-CoA carboxylase (ACC)(M80458), *Saccharomyces cerevisiae* (Sce) ACC (Q00955), *Arabidopsis thaliana* (Ath) ACC (Q13085) and human (Hsa) ACC (Q38971). Conserved residues are marked by closed circles, the canonical lysine by an asterisk above the sequence, and the mutated site by an arrow.

**Fig. 1**



**Fig. 2**



**Fig. 3**

		*↓		
Sto	114	IRVKEGDAVNKGQPLLSIEAMKSETVISAPVGGVVQKIMVKPGQGVKKGDLLLIK		169
Eco	101	AFIEVGQKVNVDGDTLCIVEAMKMNQIEADKSGTVKAILVESGQPVEFDEPLVIE		156
Sce	697	FLVENGEHIIKGPYAEIEVMKMQMPLVSQENGIVQLLKQ-PGSTIVAGDIMAIMT-		748
Ath	673	YLISDNSNIDADTPYAEVEVMKMCPLLSPASGVIHFKMSE-GQAMQAGELIANLD-		724
Hsa	748	YIVEDGGHVFAGQCYAEIEVMKMMVMTLTAVESGCIHYVKR-PGAALDPGCVLAKMQ-		799
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