Substrate specificity of archaeon *Sulfolobus tokodaii* biotin protein ligase

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Abstract

Biotin protein ligase (BPL) is an enzyme mediating biotinylation of a specific lysine residue of the carboxyl carrier protein (BCCP) of biotin-dependent enzymes. We recently found that the substrate specificity of BPL from archaeon *Sulfolobus tokodaii* is totally different from that of many other organisms, in reflection of a difference in the local sequence of BCCP surrounding the canonical lysine residue. There is a conserved glycine residue in the biotin-binding site of *Escherichia coli* BPL, but this residue is replaced with alanine in *S. tokodaii* BPL. To test the notion that this substitution dictates the substrate specificity of the latter enzyme, this residue, Ala-43, was converted to glycine. The K_m values of the resulting mutant, A43G, for substrates, were smaller than those of the wild type, suggesting that the residue in position 43 of BPL plays an important role in substrate binding.

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

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

Introduction

Biotin protein ligase (BPL) or holocarboxylase synthetase (HCS) is the enzyme mediating post-translational biotinylation of an apo form of biotin-dependent enzymes. BPL from *Escherichia coli*, studied most extensively, mediates biotinylation of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. The substrate specificity of BPL has been regarded as broad, as a BPL from one organism can usually biotinylate the BCCP domain from different organisms as long as the local sequence surrounding the receptive lysine is conserved [1-4]. We recently found that there is an exception to this rule, as BPL from thermophilic archaeon *Sulfolobus tokodaii* can biotinylate its own BCCP but not *E. coli* BCCP [5]. Likewise, BPL from *E. coli* can biotinylate its own BCCP but not *S. tokodaii* BCCP. The overall homology of the two substrate proteins is *ca.* 18% but the sequence around the canonical lysine (Lys-122 in *E. coli* and Lys-135 in *S. tokodaii)* is well conserved with a notable exception; the residue just C-terminal to the lysine is methionine (M) or related amino acids in many cases, but serine (S) occupies this position in *S. tokodaii* BCCP. It was found by characterization of mutant S136M of *S. tokodaii* BCCP and mutant M123S of *E. coli* BCCP that this substitution is at least responsible for the different substrate specificity of biotinylation. In the meantime, there is a conserved glycine (G, Gly-115) residue near the biotin-binding site of *E. coli* BPL [6], but this residue is replaced with alanine (A, Ala-43) in *S. tokodaii* (Fig. 1) [7]. It seemed probable that this substitution is complementary to the substitution of less bulky serine in *S. tokodaii* BCCP for the methionine in *E. coli* BCCP and is associated with the substrate specificity of BPL. This notion was assessed herein by mutagenesis of Ala-43 into glycine. Besides showing no cross-reactivity with *E. coli* BPL, *S. tokodaii* BPL possesses another unique characteristic; it releases the product, biotinylated BCCP or holo BCCP, very slowly. This phenomenon was also explored to some depth in this article.

Materials and methods

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). Vector pET24a and *E. coli* BL21(DE3) strain 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 DNA sequencing kit, CEQ^{TM}DTCS quick start kit, was from Beckman Coulter (Fullerton, CA). [^{3}H]Biotin (specific activity 39.0 Ci/mmol) was obtained from Amersham (Piscataway, NJ). Other common chemicals were obtained from local suppliers.

Construction of an over-expression plasmid for A43G mutant of *S. tokodaii* BPL

An over-expression plasmid for A43G mutant of *S. tokodaii* BPL was prepared by two-step PCR according to the procedure described previously [5]. Thus, upstream and downstream regions relative to the site of mutation, Ala-43, were amplified separately with pstBPL as template and the following combinations of primers, P-pET24a and stBPL-B2, stBPL-F2 and stBPL-B1 (Table 1), where pstBPL is an over-expression plasmid for *S. tokodaii* wild-type BPL and P-pET24a represents part of the vector sequence. PCR conditions were as follows: 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 two fragments thus obtained were recovered and allowed to anneal and extend. The second PCR was run with a combination of stBPL-F1 and stBPL-B1, and the product was TA cloned into TOPO vector, sequenced and re-cloned into pET24a to give pstBPL(A43G).

Protein purification

E. coli BL21(DE3) transformed with pstBPL(A43G) was grown on the Luria-Bertani (LB) plate supplemented with 50 μg/ml kanamycin. A fresh overnight culture (10 mL) from a single colony was used to inoculate 1 L of medium. The culture was grown at 37 °C for 8-10 h, then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was incubated for an additional 8-10 h. The protein was purified according to the procedures described previously [5]. In brief, the harvested cells were disrupted by sonication and centrifuged, and the supernatant was subjected to ammonium

sulfate fractionation, DEAE cellulose, and butyl Toyopearl chromatography (Tosoh, Tokyo, Japan). Typically, 10 mg of A43G mutant of *S. tokodaii* BPL was obtained from a 1 L culture.

Steady state kinetic analysis of biotinylation

The activity of *S. tokodaii* wild type and mutated BPLs was determined by measuring the incorporation of [^{3}H]biotin into either wild type or the mutated apo BCCP as described previously [5,8]. Briefly, unless otherwise stated, the assays contained 50 mM Tris-HCl, pH 8.0, 0.5 mM ATP (*S. tokodaii* wild type BPL), 0.1 mM ATP (*S. tokodaii* mutated BPL), 5.5 mM $MgCl_2$, 5 μM biotin, 50 nM [^{3}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* wild type BCCP; 2 – 100 μM *S. tokodaii* mutated BCCP or *E. coli* wild-type and mutated BCCPs. The reaction was initiated by addition of purified 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.

High performance gel filtration chromatography

A reaction mixture of *S. tokodaii* BPL and BCCP was analyzed by high performance gel filtration chromatography on a TSK gel G3000SWXL column (Tosoh) [9]. The biotinylation reaction was conducted under the same conditions as those for the kinetic analysis at 37 °C for 30 min with 5 μM BPL and 10 μM apo BCCP. Twenty μl of the reaction solution were applied to the column and eluted at a flow rate of 0.5 mL/min using a mobile phase of 100 mM potassium phosphate buffer (pH 7.0) containing 100 mM Na_2SO_4 and the eluted samples were monitored at 210 nm. The gel filtration column was calibrated with a set of proteins (Amersham Biosciences). The apparent molecular masses of the

samples were estimated from the calibration curve obtained.

Results

Characteristics of the biotinylation system of *S. tokodaii*

One of the characteristic features of the biotinylation system of *S. tokodaii* is that the product, holo BCCP, dissociates from the enzyme only slowly. In fact, the reaction virtually ceases at 37 ˚C where one equivalent of apo BCCP is biotinylated (Fig. 2). As the temperature is raised, the reaction starts to turn over more quickly and hence some of the kinetic experiments were carried out at 70 ˚C. Incidentally, the proteins do not seem to denature even at temperatures as high as 80 ˚C, as a good linearity was secured in the Arrhenius plot for the biotinylation of *S. tokodaii* wild type BCCP over the 37 – 80 ˚C range with a correlation coefficient 0.994 for the rates at 37, 50, 60, 70 and 80 ˚C. The energy of activation obtained from the slope of the line was 125 kJ/mol, a value unusually large for an enzymic reaction, presumably because a product release process as well as the catalytic process is contained in it. By contrast, slow release of the product was not observed with mutant S136M of *S. tokodaii* BCCP as substrate. The Michaelis constant K_m for the mutated BCCP was larger (see below), indicating that the residue in position 136 of *S. tokodaii* BCCP somehow affects its binding to BPL. The energy of activation for mutant S136M was estimated to be 73 kJ/mol from a similar plot for the rates at the same temperatures with a correlation coefficient of 0.998.

The k_{cat} for the wild type BCCP at 70 ˚C was 40 times smaller than that for the mutant S136M (Table 2), suggesting that dissociation of the product from enzyme still limits the overall rate. By contrast, the K_m for wild type BCCP is 200 times smaller than that for mutant S136M, thereby compensating the adversity in the catalytic process.

The notion that holo BCCP remains bound by BPL is supported by the following HPLC experiments. A reaction mixture of BPL (calculated molecular mass of 26,620 Da) and apo BCCP (16,798 Da) at a 1 to 2 molar ratio was subjected to gel filtration HPLC along with the control sample without biotin. BPL and apo BCCP were eluted separately at 19.8 and 21.1 min, respectively, in the control, showing that the two do not form a tight complex under

the experimental conditions. By contrast, the peak for BPL disappeared in the sample with biotin, and instead a new peak was observed at 18.9 min, a position of higher molecular mass. The mass of 46 kDa for that peak deduced from a calibration curve suggests a composition of $(BPL)_1$(holo BCCP)$_1$ (43,644 Da) (Fig. 3). The identity of the complex was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis for the 18.9 min peak (data not shown), indicating that BPL and holo BCCP form a tight complex.

In addition to the tight binding of holo BCCP to BPL, substrate inhibition was observed for ATP in the biotinylation system of *S. tokodaii*. Hence, kinetic data were analyzed by taking this into account [10,11]. The K_I values thus obtained for wild type and A43G mutant of BPL are larger than the K_m for ATP (Table 3), suggesting that the inhibitory binding of ATP is weaker than the productive one. It is not known whether the productive and inhibitory bindings occur at the same (active) site.

Kinetic behavior of mutant A43G of *S. tokodaii* BPL

It was found that mutant A43G of *S. tokodaii* BPL is one half as reactive as wild type BPL with *S. tokodaii* BCCP as substrate at 70 ˚C, suggesting that the residue in position 43 does not affect the catalysis of BPL significantly. Mutant S136M of *S. tokodaii* BCCP served as a poorer substrate for the mutated BPL than wild type BCCP, mainly because the K_m is much larger than that of the wild type substrate, while k_{cat} compensated for this adversity to a considerable extent (Table 2). The observation that the K_m of the mutated enzyme for S136M BCCP is much larger than that for wild type BCCP was totally contrary to our expectation, nevertheless it suggests that the residue in position 43 of *S. tokodaii* BPL somehow affects binding of apo BCCP, as the K_m of the mutated enzyme for the S136M mutant of BCCP is smaller considerably than that of the wild type enzyme.

In addition, affinity of A43G mutant of BPL for biotin and ATP was enhanced considerably from that of wild type BPL with S136M mutant of BCCP as co-substrate (Table 3). Thus, its K_m values for biotin and ATP were four and 25 times smaller, respectively, than those of wild type BPL. Given the spatial disposition of biotin bound at the active site of *E. coli* and *pyrococcus horikoshii* BPL in the crystal state [6,12] is virtually retained during

biotinylation of BCCP in *S. tokodaii*, the reaction center is to be located rather far away from position 43 of the enzyme. It is suggested that some kind of long-range effect is operating but the detailed mechanism remains to be clarified.

E. coli BCCP was not biotinylated at all by mutant A43G of *S. tokodaii* BPL, but its mutant M123S was now biotinylated to a measurable extent (Table 2). This observation was also contrary to our expectation. The substrate activity of mutant M123S was higher with A43G mutant than with the wild type enzyme, mainly because its K_m was smaller three times.

Discussion

E. coli BPL was studied extensively by *in vivo* mutagenesis and among a number of mutants showing various phenotypes is a G115S mutant, which requires high concentration of biotin in the medium for survival, suggesting that the biotin binding capability was impaired in that mutant [13]. Gly-115 of *E. coli* BPL is replaced with more bulky alanine (Ala-43) in *S. tokodaii* and two other members of *Sulfolobaceae*, *S. acidocaldarius* and *S. solfataricus*. As Gly-115 is located near the ureido group of biotin bound at the active site of *E. coli* and *P. horikoshii* BPL [6,12], it may play an important role in substrate binding and subsequent biotinylation. This notion was assessed by converting Ala-43 of *S. tokodaii* BPL to glycine. The observation that the K_m of mutant A43G of *S. tokodaii* BPL for biotin is smaller than that of the wild type is consistent with the phenomenon observed in *E. coli*, as mutant A43G of *S. tokodaii* BPL is equivalent in a sense to the wild type BPL in *E. coli*. Hence, it would not be surprising to see that the K_m of *S. tokodaii* BPL with alanine in position 43 for biotin was larger than that of the mutant with glycine there.

Furthermore, we envisaged that the substitution of alanine for the glycine in *S. tokodaii* BPL may be complementary to the substitution of less bulky serine in *S. tokodaii* BCCP for methionine in *E. coli*. The results obtained were, however, totally contrary to our prediction; the K_m of A43G mutant of BPL for the S136M mutant of BCCP was larger significantly than that for wild type BCCP. This happened, presumably because the mutated BPL still retains high affinity for wild type BCCP. Nonetheless, the fact that the

K_m of the mutated enzyme for the S136M mutant of BCCP was smaller considerably from that of the wild type enzyme supports the notion that Ala-43 of *S. tokodaii* BPL affects the binding of BCCP.

One of the notable features of the biotinylation system in *S. tokodaii* is that the product (holo BCCP) release from the enzyme is slow. This happens because the affinity of BPL for holo BCCP is extremely high and this phenomenon may be understandable from the fairly small K_m values of BPL for biotin and apo BCCP (Tables 2 and 3). The energy of activation for wild type BPL with wild type and the mutated BCCPs as substrate was 125 and 73 kJ/mol, respectively. Assuming that the energy of activation for the biotinylation is identical in the two substrates, as revealed by the absence of slow release with the mutated BCCP as substrate, the difference in the energy of activation (52 kJ/mol) may be taken as representing the energy for the dissociation of wild type holo BCCP from the enzyme active site (ΔG). The dissociation constant (K_d) estimated from this energy by means of $\Delta G = -RT \ln K_d$, where R and T represent the gas constant and absolute temperature, respectively, is 1.1×10^{-9} M at 30 ˚C. It is not certain whether the dissociation constant of this magnitude warrants extremely slow release of the product. Nor is it known why slow release of holo BCCP is observed only in *S. tokodaii*; in fact, such a phenomenon was not observed even in thermophilic *Aquifex aeolicus* [4].

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Figure legends

Fig.1. Alignment of the partial amino acid sequences of BPL from selected organisms: Sto, *S. tokodaii* (NCBI protein sequence database accession number **ST1525**); Eco, *E. coli* (**P06709**); Sce, *Saccharomyces cerevisiae* (**P48445**); Ath, *Arabidopsis thaliana* (**P92975**); Hsa, humans (**P50747**). Conserved residues are marked by closed circles (●) and the mutated site by an open circle (○).

Fig. 2. Time course of product formation in the biotinylation reaction of *S. tokodaii* apo BCCP by *S. tokodaii* BPL at 37 ˚C. The reactions were carried out with 1 (●) or 2 μM (○) *S. tokodaii* BPL in the presence of 100 μM *S. tokodaii* apo BCCP, 0.50 mM ATP, 5.5 mM magnesium chloride and 5.0 μM biotin.

Fig. 3. Estimation of the molecular mass of *S. tokodaii* BCCP, BPL and the complex of holo BCCP with BPL by gel filtration chromatography on TSK G3000SWXL. The standard proteins (○) used for constructing the calibration curve were ribonuclease A (13.7 kDa), chymotrypsinogen A (23 kDa), ovalbumin (43 kDa), and albumin (67 kDa). Elution of *S. tokodaii* BPL, BCCP and the complex of holo BCCP with BPL is represented by closed circles (●).

Figure 1

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Sto   1  MMCISMLIFKFPSVTSTQDLAEAIYQIINADE---FVIVAEEQTRARGRYKREWYSPKGG-  56
Eco  74 -GQLDGGSVAVLPVIDSTNQYLLDRIGELKSG----DACIAEYQQAGRGRRGRKWFSPFGA- 129
Sce 377 -QNTIGSLLLYGEVVTSTSTILNNNKSLLSSIPESTLLHVGTIQVSGRGRGGNTWINPKGV- 436
Ath 107 -THRFGRFLIWSPRLSSTHDVVSHNFSELPVG----SVCVTDIQFKGRGRTKNVWESPKGC- 162
Hsa 462 -TKQLGKVILFAEVTPTTMRLLDGLMFQTPQEMG--LIVIAARQTEGKGRGGNVWLSPVGC- 519
                    •                                •  ○ ••    •  •  •
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Figure 2

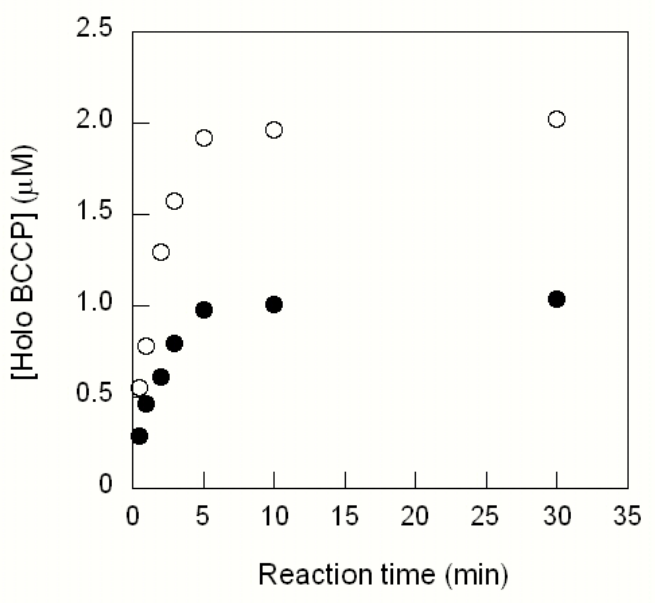

Figure 3