The Biotinyl Domain of *Escherichia coli* Acetyl-CoA Carboxylase

EVIDENCE THAT THE “THUMB” STRUCTURE IS ESSENTIAL AND THAT THE DOMAIN FUNCTIONS AS A DIMER*

Received for publication, July 6, 2001
Published, JBC Papers in Press, August 8, 2001, DOI 10.1074/jbc.M106353200

John E. Cronan, Jr.§

From the Departments of Microbiology and Biochemistry, University of Illinois, Urbana, Illinois 61801

Biotin carboxyl carrier protein (BCCP) is the small biotinylated subunit of *Escherichia coli* acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the first committed step of fatty acid synthesis. Similar proteins are found in other bacteria and in chloroplasts. *E. coli* BCCP is a member of a large family of protein domains modified by covalent attachment of biotin to a specific lysine residue. However, the BCCP biotinyl domain differs from many of these proteins in that an eight-amino acid residue insertion is present upstream of the biotinylated lysine residue. X-ray crystallographic and multidimensional NMR studies show that these residues constitute a structure that has the appearance of an extended thumb that protrudes from the otherwise highly symmetrical domain structure. I report that expression of two mutant BCCPs lacking the thumb residues fails to restore growth and fatty acid synthesis to a temperature-sensitive *E. coli* strain that lacks BCCP when grown at nonpermissive temperature. Alignment of BCCPs from various organisms shows that only two of the eight thumb residues are strictly conserved, and amino acid substitution of either residue results in proteins giving only weak growth of the temperature-sensitive *E. coli* strain. Therefore, the thumb structure is essential for the function of BCCP in the ACC reaction and provides a useful motif for distinguishing the biotinylated proteins of multisubunit ACCs from those of enzymes catalyzing other biotin-dependent reactions. An unexpected result was that expression of a mutant BCCP in which the biotinylated lysine residue was substituted with cysteine was able to partially restore growth and fatty acid synthesis to the temperature-sensitive *E. coli* strain. This complementation was shown to be specific to BCCPs having native structure (excepting the biotinylated lysine) and is interpreted in terms of dimerization of the BCCP biotinyl domain during the ACC reaction.

Acetyl-CoA carboxylase (ACC) catalyzes the first step in fatty acid synthesis, the synthesis of malonyl-CoA from acetyl-CoA (1). ACC is a biotin-dependent enzyme, and like all biotin enzymes, the cofactor must be covalently attached to ACC for enzyme activity. Biotin-dependent carboxylases are found in several molecular forms (2, 3). In *Escherichia coli* and many other bacteria, the functional enzyme seems to consist of two copies of each of four different subunits (4–6). The biotin cofactor is attached to the biotin carboxyl carrier protein (BCCP) subunit, the sole biotinylated protein of this organism (Fig. 1). In the first partial reaction, the heterocyclic ring of the attached biotin moiety is carboxylated by the biotin carboxylase subunit. The carboxyl group is then transferred from biotin to acetyl-CoA to produce malonyl-CoA by the carboxyltransferase, a complex of two proteins called ₦ and ₦. Similar ACCs (referred to as heteromeric ACCs) are found in the chloroplasts of many plants (7). In contrast, the ACCs found in mammals, fungi, and plant cytosol are composed of a single very large protein that includes sequences homologous to the four *E. coli* ACC subunit proteins. Other biotin-dependent enzymes have structures intermediate between these extremes. For example, the propionyl-CoA carboxylase of mammalian mitochondria (8) as well as those of mycobacteria and Streptomycetes are composed of two proteins. One protein contains the carboxyltransferase sequences, whereas the second has the biotin carboxylase active site and a biotin domain.

In virtually all biotinylated proteins, the site of biotin attachment is a lysine side chain found at the center of a strongly conserved sequence of ~70 amino acid residues located at the carboxyl terminus of the proteins (9). Based on the conservation of key glycine residues and stretches of hydrophobic residues, Brocklehurst and Perham (10) proposed that this sequence comprised biotinyl domains that would have structures very similar to those determined for the lipoic acid-modified domains of pyruvate dehydrogenases (lipoyl domains). This prediction was borne out by the structures of the biotinyl domains of the *E. coli* acetyl-CoA subunit BCCP (also called AccB) (11–14) and, more recently, that of the *Propionibacterium shermanii* 1.3 S transcarboxylase subunit (15). Extensive structural studies of the *E. coli* BCCP biotinyl domain have been reported. An x-ray crystal structure of the holo (biotinylated) form of the protein (11) was followed by four NMR structures: two each of the holo and apo forms of the protein (12–14). In the first biotinyl domain structure, Athappilly and Hendrickson (11) showed that the domain is a remarkably symmetrical structure of two sets of four antiparallel ₦-strands (see Fig. 2). However, the symmetry of the molecule is disrupted by a protruding eight-residue structure called the “thumb,” which in the crystals was found to be hydrogen-bonded to the uriedo ring of the biotin moiety. These authors pointed out that the thumb insertion is not found in other biotinylated proteins, suggesting an insertion or a deletion of these residues during the evolution of biotinyl domain structures. (I will refer to the thumb as an insertion despite the ambiguity.) Alignment of the *E. coli* BCCP biotinyl domain sequence with those of lipoyl and other biotinyl domains sug-
covalently bound biotin of BCCP carries the carboxylate moiety.

BCCP is encoded by the shorter between the proteins, the structures align closely

The subsequent NMR analyses showed that the solution structure of the BCCP domain is essentially the same as the crystal structure. Moreover, the apo and holo forms of the protein were shown to differ only in that side chain packing is more favorable in the holo form (12), a finding consistent with the known greater stability of the holo form toward chemical modification and proteolysis (16). Although interaction of the thumb and the biotin moiety such as that found in the crystal structure was also observed in solution, the hydrogen bonds seen in the crystal structure could not be confirmed in solution (12). The predicted lack of thumb structure in other biotinylated proteins (11) was recently confirmed by the structure of the P. shermanii 1.3 S biotinoyl domain (15). The 1.3 S structure has a folding pattern essentially identical to that of the E. coli BCCP biotinoyl domain lacking the thumb (see Fig. 2).

What is the function of the protruding thumb of the BCCP biotinoyl domain? A role in biotinylation of the protein by the cognate BirA biotin-protein ligase seemed very unlikely since expression in E. coli of the genes encoding several "thumb-less" heterologous biotinylated proteins resulted in highly biotinylated proteins (8, 17, 18). Moreover, a BCCP biotinoyl domain deleted for the thumb residues was recently found to be efficiently biotinylated (19); and, thus, a role for the thumb in biotinylation is precluded. As will be further discussed below, the thumb insertion appears only in a subset of the biotinylated proteins (11) was recently confirmed by the structure of the BCCP biotinoyl domain lacking the thumb (see Fig. 2). The unexpected observation was made, that a protein lacking the biotinylated lysine partially complemented growth of the temperature-sensitive BCCP mutant.
had been modified by removal of the EcoRI and NcoI sites by oligonucleotide-directed mutagenesis (as described above) using oligonucleotides 13 and 14. Each of the mutant gene sequences was confirmed by DNA sequencing of both strands done by the Keck Genomics Center of the University of Illinois.

**Measurement of Fatty Acid Synthesis**—Exponentially growing cultures (0.8 or 1 ml) growing in RB medium plus the appropriate antibiotics were labeled with 5 μCi of sodium [1-14C]acetate (55 mCi/mmol; American Radiolabeled Chemicals) as previously described (4) in a 15-ml plastic centrifuge tube for the time intervals given. Labeling was terminated by addition of trichloroacetic acid to a final concentration of 5%, followed by incubation on ice for 0.5–2 h. The suspensions were then quantitatively transferred to a 1.5-ml microcentrifuge tube using a 0.5-ml wash of the original tube with 5% trichloroacetic acid; the precipitate was pelleted by centrifugation; and any remaining solution was removed. The pellets were then extracted with 0.05 ml of methanol.

**Table I**

| Oligonucleotide | Sequence |
|-----------------|----------|
| 1               | 5'-CGCCCAAGTGTTAAGCCGCTGGACCACTTCTGGATGAAACACGCGCCTCAGCGCTTGG |
| 2               | 5'-CACTTTGAGCTAGTTCGGCGCAGTACGGGCGGACGTCCGCTGGGATCTGGT |
| 3               | 5'-GATCTTCTACTCCGGGCACTTCACTGCAAGTGGGTCCGCTGGGATCTGGT |
| 4               | 5'-CCACGTTTACGCGCTGGACCACTTCTGGATGAAACACGCGCCTCAGCGCTTGG |
| 5               | 5'-AACGGCGGAGTACCGCTGGGATCTGGT |
| 6               | 5'-GGTACTTTCTACCGCGGCACTTCACTGCAAGTGGGTCCGCTGGGATCTGGT |
| 7               | 5'-GACCGTACGAGTTCGCTGGCGCAACTTCTGGCGAAAGTAGCGAGC |
| 8               | 5'-CGGGCTCAGACCAGTTCGCTGGCGCAACTTCTGGCGAAAGTAGCGAGC |
| 9               | 5'-CATGGAAGACCCCGGCAACAGCGCGGAATCTGGTACATGGGAG |
| 10              | 5'-GAGCTACTGATCACCAGTTCTGGATGAGGAGCGCTGGGATCTGGT |
| 11              | 5'-AACGCGGAGTACCGCTGGGATCTGGT |
| 12              | 5'-GTACGGTAGTTCGCTGGGATCTGGT |
| 13              | 5'-AGCTACTGATCACCAGTTCTGGATGAGGAGCGCTGGGATCTGGT |
| 14              | 5'-CGGGCTGCAGAACGAGTTCGCTGGGATCTGGT |
| 15              | 5'-CCGCGCAGACCCAGGCGCTGGGATCTGGT |
| 16              | 5'-GATACGGTAGTTCGCTGGGATCTGGT |

**FIG. 2.** Structures of the *E. coli* BCCP and *P. shermanii* 1.3 S biotinyl domains. A shows the structure of the *E. coli* domain (11) with the location of the mutation causing temperature sensitivity given, and B is the structure of the *P. shermanii* domain (15). At the bottom is a structure-based alignment done with Version 3.5 of the Swiss-PDP Viewer. The differences in root mean square distance between the two structures are symbolized as follows: *, ≤0.8 Å; †, ≤1.5 Å; ‡, ≤2.0 Å. The residues of both proteins upstream of the 12th residue of the BCCP sequence shown are the ends of the hinge regions. These residues were aligned only by sequence since the structures of the hinge regions are disordered in solution (11–14). Ec ACC, *E. coli* acetyl-CoA carboxylase; Ps TC, *P. shermanii* transcarboxylase.
RESULTS

Properties of the G133S BCCP Mutation—Strain L8 (previously called strain LA2-22) was isolated in a selection targeted to mutants having a temperature-sensitive defect in fatty acid synthesis (21). The strain was shown to grow and synthesize fatty acids normally at 30°C; but upon shift to 38°C, fatty acid-synthesizing ability was rapidly lost, and growth ceased within one cell doubling (21). Subsequent enzymatic work localized the defect to acetyl-CoA carboxylase; and although the results reported were ambiguous, the strain was found to contain decreased levels of BCCP, the biotinylated form of AccB, when grown at 30°C (22). More recent work established the G133S mutation (allele accB22) by DNA sequencing (5) and confirmed that the mutant biotinoyl domain was a defective biotinylation substrate both in vivo and in vitro (5, 24). The recent structures of the AccB biotinoyl domain (11–14) showed that Gly133 is a key residue in the turn linking β-sheets 5 and 6, and modeling showed that steric clashes result upon substitution with larger residues (excepting Ala). The biotinoyl domain of the G133S mutant protein was overproduced and purified and found to be a defective biotinylation substrate in vivo and to be abnormally sensitive to proteolysis by trypsin (24). Proteolysis in vivo also seemed possible since proteolytic products were found in the earliest purification steps (24).

In this work, the in vivo stability of G133S BCCP was examined by [3H]biotin labeling. In good agreement with prior work (21, 22), cultures grown at the permissive temperature of strain CY1336 contained only 25% of the normal level of BCCP (wild-type strain CY1375 contained 3 pmol of BCCP/10^9 cells). Labeling cultures with [3H]biotin followed by a chase with excess unlabeled biotin showed that the mutant protein was only unstable at 30°C. G133S BCCP had a half-life of ~17 h, whereas, in agreement with prior data (5), the parental BCCP strain showed no detectable turnover. Upon shift to a nonpermissive temperature (38°C), the mutant BCCP protein was rapidly degraded (half-life of ~20–25 min), whereas the wild-type protein was completely stable. The instability of G133S BCCP resulted in the BCCP concentration of strain L8 drop-}

E. coli BCCP

at 42°C for 1 h, followed by addition of 0.1 ml of chloroform. Aliquots of these solutions were then spotted onto Silica Gel G thin-layer plates (which were not activated so that the three major phospholipids of E. coli would co-migrate), which were developed in chloroform/methanol/acetic acid (65:25:8, by volume). The lipids were located by autoradiography and/or iodine staining, and the appropriate areas of silica gel were then scrapped into scintillation vials and counted in Bio-Safe II scintillation solution (Amersham Pharmacia Biotech).

Measurement of Protein Biotinylation—The levels of protein biotinylation were determined by labeling growing cultures with [8,9-3H]biotin (21, 22), cultures grown at the permissive temperature of strain L8 grown at 30°C, 38°C (28), in strain L8 at 37–38°C. Although at 42°C, fatty acid supplementation fails to allow growth (21). This is due to residual fatty acid synthesis at 37–38°C, which is blocked at 42°C. This remaining fatty acid synthesis is required to produce the 3-hydroxytetradecanoyl-acyl carrier protein of the essential lipid A component of the outer membrane (29). The three defects of the mutant protein observed at the nonpermissive temperature (degradation, feeble biotinylation, and loss of function in malonyl-CoA synthesis) are different reflections of loss of the native BCCP biotinoyl domain structure. Thus, at nonpermissive temperatures, this combination of defects results in the G133S mutation behaving as a null mutation at the protein level. Note that neither supplementation of the medium with high concentrations of biotin nor overexpression of the BirA biotin-protein ligase (or both) had any detectable sparing effect on the mutant phenotype. It should be also noted that supplementation of the medium with a mixture of saturated and unsaturated fatty acids allows growth of strain L8 at 37–38°C, although at 42°C, fatty acid supplementation fails to allow growth (21). This is due to residual fatty acid synthesis at 37–38°C, which is blocked at 42°C. This remaining fatty acid synthesis is required to produce the 3-hydroxytetradecanoyl-acyl carrier protein of the essential lipid A component of the outer membrane (29).

Construction and Expression of the Mutant BCCPs—Two mutant accB genes encoding altered BCCPs lacking the thumb sequence were constructed. In the first mutant, called ΔTh-1, the seven residues (TPPSDAK) of the thumb were replaced with a single alanine residue (which molecular modeling suggested would relieve an unfavorable Ramachandran angle in the model). Mutant ΔTh-2 is the LD mutant biotinoyl domain of Reche and Perham (19) converted to full-length BCCP. (In this construct, the seven thumb residues were simply deleted.) The two constructs were essentially indistinguishable in their properties. In should be noted that to facilitate other experiments, ΔTh-1 BCCP contains four additional mutations: four of the biotinoyl domain lysine residues were converted to arginine. (Only the lysine that becomes biotinylated remained.) Since the “wild-type” form (called 4K-4R) of the four lysine-to-arginine BCCP was indistinguishable from the wild type in vivo, these substitutions appear silent. We also tested three other biotinoyl domain mutants of Reche and Perham (19) after conversion to full-length BCCPs.

Each of the modified accB genes was cloned into a derivative of plasmid pProLar.A122 such that they would be transcribed from the synthetic ara/lac promoter (20) of the vector (the native promoter was deleted) and translated using the native accB ribosome-biding site. This vector was chosen due to its low copy number and large range of expression levels (20). In strains that overexpressed the LacI repressor and were also defective in lactose transport, addition of 75 μM IPTG gave a level of BCCP production that closely matched the normal level of expression (see below). To test the function of the various mutant BCCPs, a plasmid encoding each of the mutant BCCPs was transformed into derivatives of strain L8 and its parental strain (AB1623), which were called CY1336 and CY1357, respectively. Both host strains overproduced LacI in order to regulate BCCP expression and BirA to aid in protein biotinylation. In some cases, the medium was supplemented with biotin to assist biotinylation, although this had no effect.

Ability of the Mutant BCCPs to Support Growth of the G133S Temperature-sensitive accB Mutant—The sensitivity of the complementation activity assay for BCCP function was established using the G133S strain CY1336, carrying a plasmid encoding wild-type BCCP. In the absence of IPTG, this strain grew at ~5% of the rate observed in the presence of 75 μM IPTG.
at 37 °C. Growth rates were determined by measuring increases in the diameters of colonies formed on solid medium. In the uninduced cultures, the use of solid medium prevented the overgrowth of the cultures by revertants of the G133S mutation. (Revertants have a much faster growth rate at 37 °C than uninduced cells and rapidly overgrow liquid cultures.) Under these conditions, [3H]biotin labeling (see below) showed that the uninduced cultures contained only 7.9 ± 0.3% (−0.24 pmol/10^9 cells) of the BCCP level of wild-type strains. Therefore, these results set a lower limit for the level of BCCP required for growth of the G133S mutant at 37 °C.

Growth at nonpermissive temperatures of strain CY1336 transformed with plasmids encoding the various mutant BCCPs was tested. Fig. 3 illustrates the growth behavior of some of these strains, and the data are summarized in Table II. All of the strains grew normally at 30 °C, but only those strains that expressed wild-type (or 4K-4R), Q126E, or SMEP BCCPs grew well at 37 °C (Fig. 3 and Table II). In one case, the

### Table II

**Growth at nonpermissive temperatures**

The accB22 (G133S BCCP) strain CY1336 was transformed with each of the expression plasmids and streaked for single colonies on RB plates containing 75 μM IPTG plus the antibiotics needed for plasmid maintenance. Growth was scored after 30 h at the given temperatures. The strains were streaked for single colonies to allow growth to be distinguished from the appearance of spontaneous revertants of the G133S mutation. Those strains that grew well also showed barely detectable growth in the absence of IPTG (Fig. 3). The other strains showed no growth in the absence of IPTG. All strains grew normally at 30 °C in the presence or absence of IPTG.

| Mutation on plasmid | 37 °C | 42 °C |
|---------------------|------|------|
| None (wild-type)    | ++++ | +++ |
| 4K-4R (“wild-type”) | ++++ | +++ |
| G133S               | 0    | 0    |
| Q126E               | ++++ | +++ |
| SMEP                | ++++ | +   |
| DASMEP              | 0    | 0    |
| ΔTh-1               | 0    | 0    |
| ΔTh-2               | 0    | 0    |
| P97S                | +    | 0    |
| Y92A                | ++   | 0    |
| Y92F                | ++++ | +   |
| K122C               | +    | 0    |
| K122C/G133S        | 0    | 0    |
| K122R               | ++   | 0    |
| K122R/G133S        | 0    | 0    |

DASMEP mutant, the inability of the mutant accB gene to complement the G133S mutation could be attributed to a lack of biotinylase activity. However, in the case of the two constructs (ΔTh-1 and ΔTh-2) lacking the thumb residues, the biotinylase proteins were present at levels >5-fold greater than that needed for function of the wild-type protein (see above and Figs. 4A and 5A); and thus, the ACC function of these proteins was not limited by biotinylase levels. Each of the mutant BCCPs appeared stable *in vitro* since there were no signs of biotin-labeled degradation products upon SDS gel electrophoresis of whole cell extracts (Fig. 4A). Note that although mutant BCCPs defective in biotinylase could not be assayed by this method, the biotinyl domains of these proteins have been shown to be stable *in vivo* (19).2 Note that strains expressing the wild-type gene from the chromosome or from a plasmid had similar biotinylated protein levels (Fig. 5B).

**Ability of the Mutant BCCPs to Support Acetyl-CoA Carboxylase Activity**—As discussed above, the mutational defect in strain L8 is well characterized at both the physiological and molecular levels (see above) and predicts that growth at nonpermissive temperatures demonstrates that acetyl-CoA carboxylase activity has been restored. We checked this point for a subset of the constructs (Figs. 4B and 5B). Acetyl-CoA carboxylase activity was assayed *in vivo* by measuring the rate of [1-^14^C]acetate incorporation into fatty acids. Fatty acid synthesis is totally dependent on malonyl-CoA synthesis, and incorporation into fatty acids is the only known metabolic fate of malonyl-CoA in *E. coli* (30). The transformed CY1336 derivative strains were grown to early log phase at 30 °C and then shifted to 38 °C. (This temperature was used to allow direct comparison with the original work (21), although 37 and 38 °C gave identical results.) The rate of fatty acid synthesis was then measured by pulse labeling with [1-^14^C]acetate, followed by lipid extraction and quantitation of the incorporated radioactivity. In most cases, cultures grown in the presence of [3H]biotin were shifted in parallel with those to be labeled with [1-^14^C]acetate to compare BCCP levels with the rates of fatty acid synthesis.

All strains that carried wild-type, 4K-4R, or Q54E BCCP synthesized fatty acids at rates similar to that of the wild-type

2 J. Solbiati and J. E. Cronan, Jr., manuscript in preparation.
strain at 38 °C (Fig. 5B). All strains that failed to grow at the nonpermissive temperature were also defective in fatty acid synthesis at this temperature. Note that strain CY1336 expressing the ΔTh-1 or ΔTh-2 construct retained a trace of lipid-synthesizing activity, so these proteins were not totally defective in the ACC reaction. Given the exact agreement between complementation and acetyl-CoA carboxylase function as expected from the prior work (21, 22), the strains expressing the mutant proteins constructed in the latter stages of this work were scored only by their growth behavior in complementation assays.

**Mutagenesis of Conserved Thumb Residues**—The growth data of Fig. 3 showed two striking results. These were (i) the failure of the mutant proteins lacking the thumb residues to complement growth of the temperature-sensitive mutant and (ii) the unexpected finding that the expression of the K122C mutant BCCP resulted in complementation of the non-temperaturesensitive strain, which obviates the doublets seen in lanes 5 and 6. In these lanes, there is a faint upper band corresponding to full-length G133S BCCP plus an intense lower band corresponding to the shorter thumb deletion protein. (The lines in the gel are cracks resulting from the drying process.) B, lipid synthesis as measured by [14C]acetate incorporation in strain CY1336 carrying plasmids encoding wild-type and mutant BCCPs. All strains were derivatives of G133S strain CY1336 carrying plasmids encoding various BCCP species and were induced with 75 μM IPTG, except in lane 3. Lane 1 is strain CY1375 (wild-type) carrying the pCY462 vector. Lanes 2–8 are strain CY1336 carrying plasmids encoding the following BCCPs: lane 3, wild-type BCCP; lane 4, “wild-type” 4K-4R BCCP; lane 5, ΔTh-1; lane 6, ΔTh-2; lane 7, DASMEP; lane 8, K122C. The gel was overexposed to show the faint bands of the non-complemented strains. A fluorogram of the gel is shown.

**Fig. 4.** Analyses of biotinylation and lipid synthesis in strains expressing wild-type and mutant BCCPs. All analyses were of cultures grown at 30 °C in the presence of IPTG and shifted to 38 °C for 70 min ([14C]acetate labeling was done for the last 20 min of the 70-min incubation). A, SDS-polyacrylamide gel analysis of [3H]biotin-labeled proteins. Cells from 0.1 ml of [3H]biotin were boiled in SDS gel buffer and applied to 16% SDS gels as described under "Experimental Procedures." A fluorogram of the gel is shown. Lanes 1, 9, and 10 are strain CY1357, which expresses wild-type BCCP from the chromosome. A sample of [3H]biotin-labeled BCCP-87 (the biotinoyl domain studied previously) (27) was also loaded into lane 9 as a marker for degradation products. Lane 2 is strain CY1336, which encodes temperature-sensitive G133S BCCP from the chromosome. Lanes 3–8 are strain CY1336 transformed with plasmids encoding the following BCCPs: lane 3, wild-type BCCP; lane 4, “wild-type” 4K-4R BCCP; lane 5, ΔTh-1; lane 6, ΔTh-2; lane 7, DASMEP; lane 8, K122C. The gel was overexposed to show the faint bands of the non-complemented strains. The experiments were done as described in the legend to Fig. 4. A. levels of [3H]biotin-labeled proteins in strains CY1375 (wild-type BCCP) and CY1336 (G133S BCCP) carrying the pCY462 vector and in derivatives of strain CY1336 carrying plasmids encoding the BCCP species given. The data are expressed in pmol of protein-bound biotin/10^9 cells. B, [14C]acetate incorporation into the phospholipids of the same cultures. The data are expressed in percent of the value given by the wild-type strain (1013 dpm incorporated per min by 10^7 cells). The protein encoded by the plasmid-borne accB gene is denoted by p, whereas the protein encoded by the chromosomal accB gene is given after the slash. FA Syn, fatty acid synthesis; WT, wild-type.

**Fig. 5.** Biotinylation and lipid synthesis in strains expressing wild-type and mutant BCCPs. The experiments were done as described in the legend to Fig. 4. A, levels of [3H]biotin-labeled proteins in strains CY1375 (wild-type BCCP) and CY1336 (G133S BCCP) carrying the pCY462 vector and in derivatives of strain CY1336 carrying plasmids encoding the BCCP species given. The data are expressed in pmol of protein-bound biotin/10^9 cells. B, [14C]acetate incorporation into the phospholipids of the same cultures. The data are expressed in percent of the value given by the wild-type strain (1013 dpm incorporated per min by 10^7 cells). The protein encoded by the plasmid-borne accB gene is denoted by p, whereas the protein encoded by the chromosomal accB gene is given after the slash. FA Syn, fatty acid synthesis; WT, wild-type.
experiments examining the specificity of BirA, the biotin-protein ligase that attaches biotin to the Lys122/H9280-amino group (23). This mutant was included in the above experiments to act as a negative control since it cannot be biotinylated. To our surprise, expression of the K122C protein in strain L8 allowed slow growth of the strain at 37°C (Fig. 3 and Table II). The observed complementation was not due to homologous recombination between the plasmid-borne and chromosomal mutant accB genes because all of the cells formed colonies, and the mutations are separated by only 32 base pairs (which would essentially block recombination). Moreover, recombination was precluded because the complementation was partial; the strain producing K122C BCCP grew only at 37°C, but not at 42°C, whereas complementation with the wild-type gene allowed growth at both temperatures (Table II). Derivatives of strain CY1336 that expressed either the wild-type BCCP protein or a protein in which the lysine residues (excepting that normally biotinylated) had been converted to arginine grew normally at 37 or 42°C (Fig. 3 and Table II). The latter protein was included because G133S BCCP carries the same four lysine-to-arginine substitutions.

The growth of the K122C-complemented strain was measured by plating on agar medium and following increases in colony diameter with time. At 37°C, the K122C BCCP-producing strain grew at about one-fourth of the rate of the wild-type strain. For example, the K122C BCCP-producing strain required 90 h to form colonies of 1-mm diameter, whereas the wild-type strain formed 1-mm colonies in 22 h. Growth of the complemented strain at 37°C required induction. No growth was observed when IPTG was omitted from the medium, and colonies formed more rapidly when the strain was grown in the presence of IPTG at 30°C prior to plating at 37°C (Fig. 3). Solid medium was used to prevent the overgrowth of the cultures by revertants of the L8 point mutation, which often invalidated growth curves done in liquid medium.

Specificity of Complementation of the G133S Mutation—The most straightforward interpretation of the observed complementation was an interaction between the G133S and K122C BCCPs that somehow allowed function of the G133S protein in the ACC reaction. If so, this interaction should require the complementing protein to have a well folded structure. The first test of this premise was to introduce the G133S mutation into K122C BCCP to destabilize the domain structure. As expected, introduction of this second mutation to give K122C/G133S BCCP resulted in the complete loss of complementation activity (Fig. 3). An additional set of tests of complementation specificity used several of the biotin domain mutants of Reche and Perham (19). These workers altered the biotin domain to resemble the structurally very similar pyruvate dehydrogenase domains that become modified by attachment of lipoic acid. The
altered residues are located close to the site of biotin attachment and substitute the BCCP MKMMNQIE sequence with MKMMNIE (the Q126E mutant), MKMSMIEP (the SMEP mutant), or DKSASMEIP (the DASMEP mutant). (The site of covalent modification is in boldface, and the substituted residues are underlined.) All three mutant domains were reported to be modified to varying extents with lipoic acid in vivo upon supplementation of the medium with the lipoate. In the absence of lipoic acid, the Q126E and SMEP proteins were reported to be efficiently biotinylated, whereas the DASMEP protein could not be biotinylated either in vivo or in vitro (19). The restriction fragments encoding these mutant domains were ligated into the wild-type gene to give full-length BCCPs carrying the Q126E, SMEP, and DASMEP mutations. These mutant genes (and all of the other genes described below) were introduced into vector pCY462 and then into host strain CY1336 as described above. Expression of the Q126E protein complemented the thermosensitive accB mutation in a manner indistinguishable from the wild-type protein, and expression of the SMEP protein gave a strong complementation phenomenon (both proteins were biotinylated normally); and thus, neither mutant could be used to examine the K122C complementation specificity. However, upon expression of the DASMEP protein, no complementation was observed (Fig. 3 and Table II). Since both of the constructs lacking the thumb also failed to complement (Fig. 3 and Table II), these data showed that mutant biotin domains lacking native structure were unable to mimic the complementation observed upon expression of K122C BCCP regardless of whether they were (ΔTh-1 and ΔTh-2) or were not (DASMEP) biotinylated.

Finally, a second K122 construct, K122R, was made in the wild-type gene to test if the K122C mutation was a special case. Arginine substitution was a more conservative change than the K122C substitution since the lysine charge and long aliphatic side chain were retained. However, the K122R mutation was expected to block biotinylation due to the very high pKₐ and branched structure of the arginine guanidino group, and this was found to be the case. Strain CY1336 producing the K122R protein had the same low biotinylated protein content as the strain carrying the vector plasmid (data not shown). Expression of the gene in the standard vector/host combination resulted in stronger complementation than that seen with the K122C protein. The K122R BCCP-producing strain grew at about half the rate of the wild-type strain at 37 °C. However, growth at 42 °C was defective; only barely detectable growth was seen on plates incubated for 72 h. As previously observed for K122C BCCP, induction with IPTG was required for complementation, and the double mutant K122R/G133S completely lacked complementation activity (Table II).

Mechanism of the Complementation of the G133S Mutant by Lys122 Mutant Derivatives—Growth of strains expressing the K122C protein indicated that acetyl-CoA carboxylase activity had been restored to a level that permitted sufficient fatty acid synthesis for cell doubling. This was tested by assaying the rate of fatty acid synthesis by incorporation of [14C]acetate into fatty acids after shift to 38 °C as described above. As expected, production of the K122C protein partially restored fatty acid synthesis at 38 °C, whereas production of the other mutant BCCPs gave no or only barely detectable fatty acid synthesis (Figs. 4B and 5B). The K122C mutant fatty acid synthesis was restored to ~15% of the normal rate, and the restoration of fatty acid synthesis required induction with IPTG (Figs. 4B and 5B).

The K122C BCCP protein could permit growth of the temperature-sensitive mutant at 37 °C by stabilizing the G133S protein against proteolysis and thereby increasing the level of biotinylated protein present at the nonpermissive temperature. This increased level of functional BCCP would then allow growth and fatty acid synthesis. Another possibility was that the K122C protein interacts with and stabilizes the G133S protein and allows it to retain some function in the ACC reaction at 37–38 °C. The possibility of stabilization of G133S against proteolysis was tested by [3H]biotin labeling of cultures of strain CY1336 expressing K122C BCCP at 30 °C, followed by shift of the cultures to 38 °C in the presence of excess nonradioactive biotin. The level of biotinylated protein was then followed with time for several hours. Expression of the K122C protein had no evident effect on the level of cellular biotinylated protein or on the half-life of the G133S protein (data not shown). Therefore, complementation seems most likely to be explained by a protein-protein interaction that results in improved function of the G133S protein in the ACC reaction.

DISCUSSION

Function of the BCCP Thumb—Biotin-dependent carboxylases and decarboxylases are a strongly conserved protein family (2, 3), and the sequences of the biotinoyl domains of these proteins are especially conserved. However, upon alignment, the biotinoyl domains clearly fall into two classes, those that have the thumb insertion found in E. coli BCCP and those lacking this sequence (Fig. 6A). In these alignments, the thumb sequences have the same length, although they vary in sequence except at the two residues tested above. Given the strong sequence conservation and the multiplicity of reactions catalyzed by biotin-dependent enzymes, it has previously been difficult to assign a specific enzymatic reaction to a biotin-dependent enzyme based on sequence alone. However, among the biotin-dependent enzymes of known specificity, all of the ACC biotinoyl domains have the thumb sequence, whereas the thumb-less domains are from enzymes that catalyze reactions other than malonyl-CoA synthesis. Moreover, thumb-containing biotinoyl domains are found only in organisms that synthesize fatty acids. The archaea, which lack fatty acids, also lack thumb sequences, although there are a dozen or so biotinoyl domain sequences among the complete (and substantially complete) genomes. Therefore, the location and sequence of the thumb region seem to provide a motif (Fig. 6B) useful for identifying the biotin proteins of multifunctional ACCs.

What is the function of the BCCP thumb? It clearly plays no essential role in either protein biotinylation or protein stability; yet it has been retained in the multisubunit ACCs of diverse bacteria and plant plastids. The fact that the thumb structure interacts with the biotin head group suggests that the thumb may act as a mobile lid for either (or perhaps both) the biotin carboxylase or carboxyltransferase active sites. The findings that the Y92A and P97S point mutations have strong effects on the function of this rather mobile structure suggest that the thumb must have (or be able to attain) a defined structure to be effective. If the thumb acts as a lid, it seems reasonable that it functions to prevent solvent water molecules from entering an active site. Indeed, desolvation of the active site has been proposed as a mechanism to aid transfer of the CO₂ moiety of carboxybiotin to the acceptor molecule (2). If the thumb acts as a lid, the lid would have to fit tightly, and the fit might be easily disrupted by amino acid substitutions. Substitutions of residues 92 and 97 (E. coli BCCP numbering) disrupt thumb function in the ACC reaction, but is not true of the other thumb residues since the BCCPs of both Bacillus subtilis (31)³ and Pseudomonas aeruginosa (32) complement the E. coli accB

³ E. James and J. E. Cronan, Jr., unpublished data.
The active sites of biotin carboxylase are located on the monomer faces opposite those used in dimer formation and thus are relatively far apart (62 Å between the GLH288 active-site residues) (38, 40). The constraints imposed by the biotin carboxylase crystal structure suggests a simple model in which two parallel assemblies of active sites are separated by the distance (60–70 Å) between the two active sites of the biotin carboxylase dimer. The K122C complementation data suggest that paired biotinyl domains move between the biotin carboxylase and carboxyltransferase active sites. The proposed biotinyl domain-pairing interaction must be weak based on the NMR data. However, given a very high local domain concentration resulting from the tethering of the other ends of the BCCP molecules, it seems possible that interactions could be sufficiently strong to partially counter the lability of the G133S protein. The shape of the BCCP biotinyl domain suggests a possible site where interaction could occur. The domain shape is roughly that of a flattened barrel with the biotin protruding from the top (in the depiction of Fig. 1) and the amino and carboxyl termini protruding from the other (bottom) end. If the bottom ends of two domains interact, this would position the two biotin moieties ~60 Å apart, a spacing similar to that of the biotin carboxylase active sites. The G133S mutation lies at the bottom of the domain close to this putative interaction surface such that the interaction with a normally folded domain could alleviate the instability of the mutant domain. This bottom surface is rather flat and somewhat hydrophobic, which could aid interaction. It is interesting that the biotinyl domain of the P. shermanii 1.3 S protein has an analogously positioned flat surface (15).

Acknowledgments—I thank Dr. John Campbell, Dr. José Solbiati, and Ethan James for useful discussions and suggestions.

REFERENCES
1. Alberts, A. W., and Vagelos, P. R. (1972) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. 6, pp. 37–82, Academic Press, New York
2. Knowles, J. R. (1989) Annu. Rev. Biochem. 58, 195–227
3. Samols, D., Thornton, C. G., Murif, V. L., Kumar, G. K., Haase, F. C., and Wood, H. G. (1988) J. Biol. Chem. 263, 6461–6464
4. Davis, M. S., Solbiati, J., and Cronan, J. E., Jr. (2000) J. Biol. Chem. 275, 28595–28599
5. Li, S. J., and Cronan, J. E., Jr. (1992) J. Biol. Chem. 267, 855–863
6. Li, S. J., and Cronan, J. E., Jr. (1992) J. Biol. Chem. 267, 16841–16847
7. Ohlrogge, J., Pollard, M., Bao, X., Foeke, M., Girke, T., Rusanka, S., Mekhodov, S., and Benning, C. (2000) Biochem. Soc. Trans. 28, 567–573
8. Leon-Del-Rio, A., and Gravel, R. A. (1994) J. Biol. Chem. 269, 22964–22968
9. Chapman-Smith, A., and Cronan, J. E., Jr. (1999) Trends Biochem. Sci. 24, 359–363
10. Brocklehurst, S. M., and Perham, R. N. (1993) Protein Sci. 2, 626–639
11. Athappilly, F. K., and Hendrickson, W. A. (1995) Structure 3, 1407–1419
12. Roberts, E. L., Shu, N., Howard, M. J., Broadhurst, R. W., Chapman-Smith, A., Wallace, J. C., Morris, T., Cronan, J. E., Jr., and Perham, R. N. (1999) Biochemistry 38, 5045–5053
13. Yao, X., Wei, D., Soden, C., Jr., Summers, M. F., and Beckett, D. (1997) Biochemistry 36, 15089–15100
14. Yao, X., Soden, C., Jr., Summers, M. F., and Beckett, D. (1999) Protein Sci. 8, 307–317
15. Reddy, D. V., Shenoy, B. C., Carey, P. R., and Sonnichsen, F. D. (2000) Biochemistry 39, 2509–2516
16. Chapman-Smith, A., Forbes, B. E., Wallace, J. C., and Cronan, J. E., Jr. (1997) J. Biol. Chem. 272, 26017–26022
17. Cronan, J. E., Jr. (1988) J. Biol. Chem. 263, 10332–10336
18. Cronan, J. E., Jr., and Reed, K. E. (2000) Methods Enzymol. 326, 440–458
19. Reche, P., and Perham, R. N. (1999) EMBO J. 18, 2673–2682
20. Lotz, R., and Bajard, H. (1997) Nucleic Acids Res. 25, 1203–1210
21. Harder, M. E., Beacham, I. R., Cronan, J. E., Jr., Beacham, K., Honegger, J. L., and Silbert, D. F. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3105–3109
22. Silbert, D. F., Pohlan, T., and Chapman, A. (1976) J. Bacteriol. 126, 1351–1354
23. Chapman-Smith, A., Turner, D. L., Cronan, J. E., Jr., Morris, T. W., and Wallace, J. C. (1994) Biochem. J. 302, 881–887
24. Cronan, J. E., Jr. (1990) J. Biol. Chem. 265, 10327–10333
25. Raetz, C. R. (1993) J. Bacteriol. 175, 5745–5753

E. coli BCCP

(G133S) mutant despite the considerable differences among the three thumb sequences. It is interesting that the side chains of the two conserved residues show rather different dynamics in the NMR structures (12–14). Tyr206 is relatively immobile, having a root mean square deviation similar to that of the peptide backbone, whereas Pro97 is considerably more mobile, particularly in the biotinylated form of the protein. Both residues interact with the biotin moiety, although the Tyr92 aromatic ring has more extensive interactions (12).

The notion of the thumb acting as an active-site lid leads to the question of why other biotin-dependent enzymes can lack this feature since biotin-dependent enzymes have a common mechanism (2). Since the thumb is essential, it seems that a structure having the function of the thumb must reside elsewhere in other biotin-dependent enzymes, presumably neighboring the biotin carboxylase and/or carboxyltransferase active sites.

Complementation by Unbiotinylated BCCPs—The observed complementation of the temperature-sensitive G133S mutation by expression of mutant AccB proteins that cannot be biotinylated demonstrates an unanticipated complexity of the ACC reaction. The BCCP biotinyl domain has been extensively studied, and the isolated domain remains monomeric even at the very high concentrations (2–3 mM) used in the NMR structural analyses (12–14). Moreover, it is clear that the monomer is fully active in in vitro biotinylination reactions (27, 33). The lack of interaction between isolated biotinyl domains in vitro suggested a model similar to that for another swinging arm enzyme, pyruvate dehydrogenase. In pyruvate dehydrogenase, each lipoc acid-modified chain is free to interact with multiple lipoyl acetyltransferase and dihydrolipoamide dehydrogenase active sites in a more or less random fashion (34). Extension of this picture to acetyl-CoA carboxylase suggested that the BCCP biotinyl domains would move independently between the biotin carboxylase and carboxyltransferase active sites. However, the intragenic complementation data reported in this study strongly support a model in which the biotinyl domains interact during the ACC reaction.

A trivial explanation for the K122C complementation results is that BCCP molecules must dimerize to associate with the other ACC subunits and that, at high temperatures, the concentration of the unstable G133S protein falls below the threshold required for efficient dimer formation. In this view, the role of the K122C and K122R proteins would be to provide molecules to allow the few remaining G133S molecules to form dimers. However, if this were the case, then expression of the DASMEP, ΔTh-1, and ΔTh-2 proteins should also have complemented, but this was not observed. Therefore, the behavior of the K122C and K122R mutants suggests a model in which paired biotinyl domains move together between the ACC active sites.

In this model, two biotinyl domains interact such that a properly folded, albeit inactive, biotinyl domain stabilizes the biotinylated temperature-sensitive domain against loss of structure. The full-length BCCP protein is probably a dimer in solution (although the strong predilection of the full-length protein to aggregate complicates the analysis), and complexes of BCCP and biotin carboxylase have been reported, although no stoichiometry has been reported (33, 35). Biotin carboxylase is known to be a dimer in solution (5, 36, 37) and was crystallized in this form (38); and thus, it seems likely that BCCP dimerizes and then binds to a biotin carboxylase dimer. If so, then this complex should be aligned to interact with the active sites of the carboxyltransferase component. The carboxyltransferase has an αβγδ structure (6, 39) and thus seems very likely to have dyad symmetry.
E. coli BCCP

30. Magnuson, K., Jackowski, S., Rock, C. O., and Cronan, J. E., Jr. (1993) Microbiol. Rev. 57, 522–542
31. Marini, P., Li, S. J., Gardiol, D., Cronan, J. E., Jr., and de Mendoza, D. (1995) J. Bacteriol. 177, 7033–7066
32. Best, E. A., and Knauf, V. C. (1993) J. Bacteriol. 175, 6881–6889
33. Nenortas, E., and Beckett, D. (1996) J. Biol. Chem. 271, 7559–7567
34. Perham, R. N., and Reche, P. A. (1998) Biochem. Soc. Trans. 26, 299–303
35. Fall, R. R., Nervi, A. M., Alberts, A. W., and Vagelos, P. R. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1512–1515
36. Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., and Lane, M. D. (1974) J. Biol. Chem. 249, 6633–6645
37. Janiyan, K., Bordelon, T., Waldrop, G. L., and Cronan, J. E., Jr. (2001) J. Biol. Chem. 276, 29864–29870
38. Waldrop, G. L., Rayment, I., and Holden, H. M. (1994) Biochemistry 33, 10249–10256
39. Blanchard, C. Z., and Waldrop, G. L. (1998) J. Biol. Chem. 273, 19140–19145
40. Thoden, J. B., Blanchard, C. Z., Holden, H. M., and Waldrop, G. L. (2000) J. Biol. Chem. 275, 16183–16190
The Biotinyl Domain of *Escherichia coli* Acetyl-CoA Carboxylase: EVIDENCE THAT THE "THUMB" STRUCTURE IS ESSENTIAL AND THAT THE DOMAIN FUNCTIONS AS A DIMER

John E. Cronan, Jr.

*J. Biol. Chem. 2001, 276:37355-37364.*
doi: 10.1074/jbc.M106353200 originally published online August 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106353200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 24 of which can be accessed free at http://www.jbc.org/content/276/40/37355.full.html#ref-list-1