

The Biotin Carboxylase-Biotin Carboxyl Carrier Protein Complex of *Escherichia coli* Acetyl-CoA Carboxylase*

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***Escherichia coli* acetyl-CoA carboxylase (ACC) is composed of four different protein molecules. These proteins form a large but very unstable complex. Hints of a sub-complex between the biotin carboxylase (BC) and biotin carboxyl carrier protein (BCCP) subunits have been reported in the literature, but the complex was not isolated and thus the protein stoichiometry could not be determined. We report isolation of the BC·BCCP complex. By use of affinity chromatography using two different affinity tags it was shown that the complex consists of a two BCCP molecules per BC molecule. The molar ratio in the complex is the same as the ratio of the subunit proteins synthesized *in vivo*. We conclude that the complex consists of a dimer of BC plus four BCCP molecules instead of the 2BC·2BCCP complex previously assumed. This subunit ratio allows two conflicting models of the ACC mechanism to be rectified. We also report that the N-terminal 30 or so residues of BCCP are responsible for the interaction of BCCP with BC and that the BC·BCCP complex is a substrate for biotinylation *in vitro*.**

Escherichia coli acetyl-CoA carboxylase (ACC)¹ catalyzes the first committed, and rate-limiting step of fatty acid synthesis, the synthesis of malonyl-CoA (1). The overall reaction consists of two distinct half-reactions (Scheme 1) the carboxylation of biotin with bicarbonate followed by transfer of CO₂ group from carboxybiotin to acetyl-CoA to form malonyl-CoA (2, 3). To carry out this two-step reaction, ACC requires two different protein subassemblies, biotin carboxylase (BC), and carboxyltransferase (CT). A third protein, biotin carboxyl carrier protein (BCCP), carries the essential biotin cofactor covalently bound to a lysine residue located 35 residues from the carboxyl terminus. In mammals, fungi, and plant cytosols, all three ACC components reside on one polypeptide chain (4). However, in the *E. coli* form of the enzyme, which has been the subject of most mechanistic investigations, the components are a series of individual proteins that form a complex. The component proteins can be isolated from one another with the BC and CT components retaining enzymatic activity in their respective half-reactions (3, 5–7). Intermediate between these two ex-

treme forms of ACC is the mitochondrial propionyl-CoA carboxylase (which also carboxylates acetyl-CoA), which is composed of two subunits. One subunit contains the CT sequences, whereas the other consists of the BC sequence followed by BCCP sequence (8).

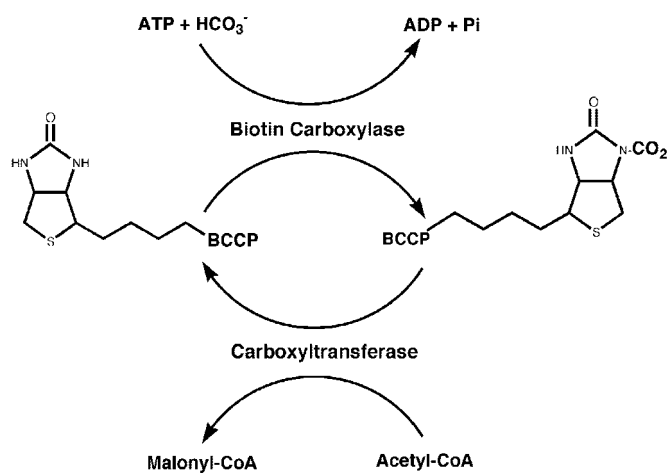
E. coli ACC seems to be a markedly unstable enzyme complex. It is thought to dissociate into sub-complexes upon undergoing the dilution that invariably accompanies cell lysis (6, 7, 9). The overall activity can be measured only when all four subunits are present at high concentrations (5, 10), although the two partial reactions, biotin carboxylase and carboxyltransferase (Scheme 1), can be measured in dilute protein solutions (3, 5). The full-length BCCP, a protein of 16.7 kDa, is a recalcitrant protein due to its very strong tendency to aggregate (1, 11). Proteolytic cleavage was shown to convert the full-length BCCP to a stable short form, which was comprised of a protease resistant C-terminal domain (1, 12, 13). This C-terminal fragment comprises the biotinylation domain, the structure of which has been determined by both x-ray crystallographic and multidimensional NMR analyses (23–26). The remainder of the protein consists of an N-terminal domain, which is involved in subunit interaction (see below). The N-terminal and C-terminal domains are connected by a proline plus alanine-rich sequence that acts as a mobile linker (18). BC, a protein of 49.4 kDa, has purified to homogeneity and shown to be a dimeric protein in solution (5, 6). The crystal structure of BC has been determined and shows a dimeric protein held together by a subunit interface arranged in a 16-stranded β -sheet (14). CT has also been purified and is composed of two protein molecules of 35 and 33 kDa in an $\alpha_2\beta_2$ arrangement (5, 15, 23), but no crystal structure is yet available. From the behavior of chloroplast enzymes, it has been suggested that a 2BC·2BCCP complex might be present *in vivo* (16). There are hints of such a complex in *E. coli* (1, 11, 13), however, it has not been clearly demonstrated and no subunit stoichiometries have been reported. Other bacterial ACCs seem more stable than that of *E. coli*. Indeed, the *Pseudomonas citronellolis* enzyme was stabilized by high salt concentrations, purified, and reported to contain two copies of each of the four subunits (9). Although this has long been considered to be the correct stoichiometry, the data are based on the intensity of Coomassie Blue staining, which varies significantly among proteins. Moreover, some variation in stoichiometry was mentioned. Therefore, the subunit stoichiometry of the active complex, which is required to understand the mechanism of the enzyme reaction, needed to be addressed.

In this report, we demonstrate the purified BC plus BCCP complex for the first time. The BC·BCCP complex has been purified by avidin plus immobilized nickel affinity chromatographic steps. Our data show that the molar ratio of BC to

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¹ The abbreviations used are: ACC, acetyl-CoA carboxylase; BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; IPTG, isopropyl- β -D-thiogalactopyranoside; CT, carboxyltransferase.



SCHEME 1

BCCP in the intact complex is 1:2 rather than the 2BCCP:2BC complex previously supposed. We also have identified the domain of BCCP required to form the BC-BCCP complex.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—ImmunoPure Immobilized Monomeric Avidin resin, Immobilized NeutrAvidin from Pierce, and nickel-nitrilotriacetic acid-agarose (Qiagen) were used for column chromatography. Most of the oligonucleotides used in this study (Table I) were synthesized by the Keck Center of the University of Illinois. Two complementary oligonucleotides used to add a hexahistidine tag to the N terminus of BC were synthesized by Integrated DNA Technologies, Inc. L-[³⁵S]Methionine and [8,9-³H]biotin were purchased from Amersham Biosciences. The media used were described previously (15, 17–19). *E. coli* BirA protein (biotin protein ligase) was purified essentially as described by Kwon and Beckett (20). Biotinylation *in vitro* was done as described previously (21).

Plasmid Constructions—Plasmid pLS182 (17) was cut with *Eco*RI plus *Bam*HI and ligated to plasmid pQE-60 from Qiagen cut with the same enzymes to give pER2. pER2 was then cut with *Eco*RI and treated with T4 DNA polymerase plus the four deoxynucleotide triphosphates to blunt the ends, followed by cutting with *Bam*HI to obtain a 3.6-kb fragment containing the *accB* (encoding BCCP) and *accC* (encoding BC) genes. This fragment was ligated to pET-14b from Novagen, which had been cut with *Xba*I treated with DNA polymerase as above, and then digested with *Bam*HI. This gave pER18, the plasmid used in the radioactive labeling experiments. The *accC* gene was amplified by PCR using pER18 as a template with primers BC-1 and BC-2 (Table I) and subcloned into pCR-2.1 from Invitrogen, resulting in pER23. Plasmid pER23 was cut with *Sac*II plus *Bam*HI and ligated to pER18 cut with the same enzymes to obtain plasmid pER24, which has a hexahistidine tag at the C terminus of AccC. Two complementary oligonucleotides, BC-N-his-F and BC-N-his-R (Table I), were combined in ligase buffer, heated to 100 °C, and allowed to slowly cool to room temperature to anneal the single strands to give a double-stranded oligonucleotide. This oligonucleotide was ligated to pER24 cut with *Age*I and *Sac*II (the oligonucleotide cassette was designed such that complementary ends would result upon annealing) to obtain pER27, in which the hexahistidine tag was added to the N terminus of AccC.

Construction of Plasmids for Deletion Analysis—Plasmid pER2 was amplified with *Pfu* Turbo DNA polymerase from Stratagene using two specially designed primers to construct deletion mutants of BCCP, according to the instructions of the manufacturer. The sequences of the oligonucleotides used for each construct are given in Table I. The odd-numbered oligonucleotides were phosphorylated with polynucleotide kinase and ATP before use. Each PCR reaction was treated with *Dpn*I to digest the template DNA, circularized by ligation, and transformed into strain TOP10F' (Invitrogen). The constructs were first analyzed by colony PCR to screen for the 12-bp deletions. Strains carrying those constructs showing the expected deletions were then induced with IPTG to allow assay of BC and BCCP expression. The constructs that survived these screens were then confirmed by DNA sequence analysis performed by the Keck Center of the University of Illinois. For the production of BCCP without concomitant overexpres-

sion of BC, the plasmids were digested with *Sac*II and then re-circularized by ligation resulting in the removal of the 2-kb *accC* fragment encoding BC.

Expression and Purification of the BC Plus BCCP Complex—Plasmid pCY216 (21) carrying the *birA* gene and plasmid pLS182 carrying the *accBC* operon were introduced into *E. coli* strain BMH71-18. Cultures were grown in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, and 2 µM biotin to an optical density of 0.8 at 600 nm. Expression was induced by addition of isopropyl-β-D-thiogalactoside to 0.5 mM and allowed to proceed for 4 h. The cells were harvested by centrifugation, resuspended in lysis buffer A, which consisted of 0.1 M sodium phosphate (pH 7.2), 0.15 M NaCl, 0.1 mM dithiothreitol for the avidin column. For the nickel chelate column lysis buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.1 mM dithiothreitol) was used. The cells were lysed by sonication. Monomeric avidin resin was treated according to manufacturer's instructions to block the "nonreversible" biotin binding sites and then added to the extract and incubated overnight at room temperature with gentle agitation. After washing with 10 volumes of lysis buffer A, the BC plus BCCP complex was eluted by incubating with 2 mM biotin in lysis buffer A at room temperature for 4 h. For deletion analysis of the BCCP N terminus, each plasmid encoding a deletion construct was introduced together with pCY216 into TOP10F' (Invitrogen) for expression of BC and BCCP proteins. Complex formation was assayed by passage through a NeutrAvidin column (Pierce Chemical Co). When NeutrAvidin was used, the incubation period was shortened to 1 h, the column was washed with 10 column volumes of 1% Nonidet P-40 in lysis buffer, and elution was accomplished by boiling the resin in SDS sample buffer. For the consecutive column purification protocol the nickel-chelate-agarose was added to the extract and incubated 1 h at 4 °C with gentle agitation. After washing the resin with 10 volumes of 25 mM imidazole in lysis buffer B, the protein was eluted with 250 mM imidazole in buffer A. The eluate was concentrated with a Centrifugal Filter unit (Millipore) having a 5000 molecular weight cut-off. Buffer A was then added, and this solution was incubated with either monomeric avidin resin or NeutrAvidin resin, and the complex was purified as described above.

Expression and Purification of the L-[³⁵S]Methionine-labeled BC-BCCP Complex—*E. coli* strain BL21(DE3)pLysS from Novagen carrying pER27 and pCY216 was grown in LB media containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, and 2 µM biotin to an optical density at 600 nm of 0.5. Protein expression was induced by addition of IPTG followed by incubated for 30 min at 37 °C. Then 0.2 µg/ml rifampicin and L-[³⁵S]methionine (3 µCi/ml) were added to the culture followed by incubation for another 30 min. The cells were then harvested, and the BC-BCCP complex was purified with the nickel chelate column and/or NeutrAvidin column as described above.

Expression and Purification of D-[8,9-³H]Biotin-labeled BCCPs—*E. coli* strain TOP10F' from Invitrogen carrying *accB* plasmids encoding the WT, Δ4, and Δ7 BCCPs plus pCY216 were grown in Rich Broth (RB) media containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, and 100 nM biotin to an optical density at 600 nm of 0.5. The cells were harvested and resuspended in RB media containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, D-[8,9-³H]biotin (2 µCi/ml), and 0.5 mM IPTG. After a 2-h incubation at 37 °C, 2 µM biotin was added to the culture and growth was continued for 2 more h at 37 °C. The cells were then harvested, and the BCCP proteins were purified with monomeric avidin resin as described above. After concentrated to 50 µl with a Centrifugal Filter Unit (Millipore) having a 5000 molecular weight cut off, the eluates were mixed with hemoglobin, and cytochrome *c* (both from Sigma) as molecular weight standards each was applied to a column (1 × 33 cm) of Sephadex G-75 equilibrated with 0.1 M sodium phosphate (pH 7.0) and 0.15 M NaCl. The flow rate was ~7 ml/h, and 0.5-ml fractions were collected. Fractions were analyzed for radioactivity using LS 6500 Multi-Purpose Scintillation Counter from Beckman Coulter. It should be noted that much of the biotinylated protein was lost before the gel filtration step presumably due to aggregation (1, 11).

RESULTS

Purification of the BC-BCCP Complex—It should be noted that, although *E. coli* BCCP is a protein of 16.7 kDa, it migrates as a protein of ~22 kDa in the standard Tris-glycine SDS electrophoresis system. This anomalous migration has been attributed to the long proline plus alanine-rich linker that lies between residues 34 and 101 (17). Indeed, small alterations of the linker have been shown to markedly alter the electrophoretic mobility of BCCP (18). In contrast, BC has an electro-

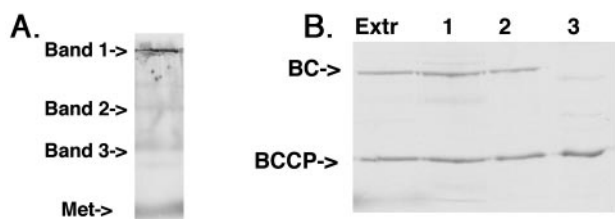


FIG. 2. SDS-PAGE analysis of BC-BCCP complex labeled with L-[³⁵S]methionine. A, BC and BCCP were co-expressed and labeled with L-[³⁵S]methionine in *E. coli* strain BL21(DE3) carrying pER18 and pCY216 as described under "Experimental Procedures" and analyzed by electrophoresis by 7.5% native PAGE. The gel was dried and exposed to the x-ray film overnight. B, the next day bands 1–3 from the gel of A were excised and rehydrated in SDS loading buffer, and the gel slices were inserted into the wells of a 12% SDS-PAGE. (The proteins were eluted from the gel slice. They were then stacked in the stacking gels and separated as usual.) The SDS-PAGE gel was then dried and analyzed with a phosphorimaging device (Fuji FLA-3000). Lane 1 is the crude extract, which had a BC to BCCP molar ratio of 1:2.1. Lane 2 is band 1 from the native gel, which had a BC to BCCP molar ratio of 1:1.95, essentially the same BC to BCCP ratio as the crude extract. Lane 3 is band 2 from the native gel, which had a ratio of BC to BCCP of 1:3.1. Lane 4 is band 3 from the native gel and contains only BCCP.

promoter to allow specific labeling of the proteins. An *E. coli* strain carrying this construct and a phage T7 RNA polymerase gene was grown until mid-log phase, and IPTG was added to induce T7 RNA polymerase synthesis. After 30 min of incubation rifampicin was added to inhibit *E. coli* polymerase activity and thereby block all protein synthesis encoded by chromosomal genes. The culture was then labeled with L-[³⁵S]methionine. This protocol was designed such that only those genes transcribed from a T7 promoter (*accB* and *accC*) would be expressed during the time that the culture was exposed to L-[³⁵S]methionine (22). The ³⁵S-labeled BC-BCCP complex was then purified through monomeric avidin column, and the eluate was loaded on an SDS-PAGE gel. As shown, in Fig. 2, two bands representing BC and BCCP were detected. The radioactivity of each band was quantitated by use of a phosphorimaging device (Fuji FLA-3000). After correction for the number of methionine residues of the two proteins (14 for BC and 9 for BCCP) the molar ratio of the two proteins was calculated. (Note that the N-terminal methionine residues of both proteins are known to be retained (17).) We obtained different ratios for the relative amounts of BC and BCCP in the crude extract and in the purified fraction. The ratio of BC to BCCP was 1:2.1 in the crude extract, whereas it was 1:2.8 in the purified fraction. We then analyzed the crude extract by native gel electrophoresis where two major bands were detected: a top band that failed to enter the separating gel and a middle band of about 160 kDa, plus smaller bands (Fig. 2). The top and the middle bands were excised and analyzed by SDS-PAGE gel electrophoresis. The ratio of BC to BCCP was 1:1.95 in the top band, whereas it was 1:3.1 in the middle band. These data suggested that the 1:2 complex was the native species and the 1:3 species was a mixture of breakdown products that had decreased BC content. Therefore, the BC-BCCP complex behaved as an unstable structure, and it seemed that there might be interactions of varying stabilities within the complex.

To approach the stoichiometry of the complex from the BC partner we constructed an operon with an altered *accC* gene that encoded an N-terminally hexahistidine-tagged BC protein. Addition of this tag has been shown not to affect the *in vitro* activity of the enzyme (23). The hexahistidine tag was inserted such that normal *accBC* operon structure and *accC* ribosome binding site were preserved (a BC construct with a C-terminal hexahistidine was also made, but the protein bound the nickel chelate column only when denatured). With this construct, the

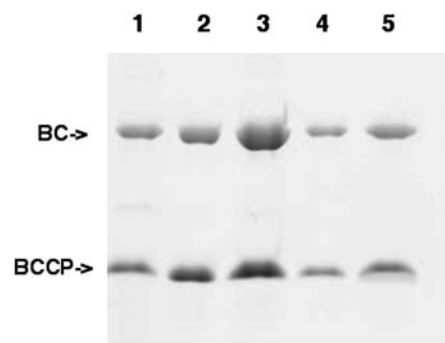


FIG. 3. Analysis of BC-BCCP complex by chromatography through immobilized avidin and/or immobilized nickel chelate columns. BC and BCCP were co-expressed and labeled with L-[³⁵S]methionine and purified through column of nickel chelate and/or NeutrAvidin columns as described under "Experimental Procedures." Each fraction was loaded onto 12% SDS-PAGE, and the radioactivity of each band was quantitated using a phosphorimaging device. Lane 1 is the crude extract where the ratio of BC to BCCP was 1:2.1. Lane 2 is the eluate from a NeutrAvidin column where the ratio of BC to BCCP was 1:3.1. Lane 3 is the eluate from a nickel chelate column and had a ratio of BC to BCCP was 1:1.42. Lane 4 (5 μ l) and lane 5 (10 μ l) are eluate fractions from the NeutrAvidin column loaded with the eluate from a nickel chelate. In these fractions the ratio of BC to BCCP was 1:2.08. The yields of BC and BCCP from the nickel chelate column were 39% and 24.7%, respectively. When these eluates were chromatographed on the NeutrAvidin column the yields of BC and BCCP were 15 and 18% of the nickel chelate column eluate, respectively. The overall yields (based on the crude extract) after passage through both columns were 4.8% and 4.5% for BC and BCCP, respectively.

BC-BCCP complex was readily purified by nickel chelate chromatography under native conditions (Fig. 3). The ratio of BC to BCCP was 1:1.42 in the eluate from the nickel chelate column. However, as in the BCCP-avidin column case, the observed ratio could have been skewed through binding of uncomplexed BC to the column. To remove any uncomplexed BC, we passed the eluate of the nickel chelate column over a tetrameric avidin column and examined the ratio of the eluate from this second column. The BC to BCCP ratio of this doubly purified preparation was 1:2.08. From the yields of radioactivity in the two proteins (given in the legend of Fig. 3) only 4.5–4.8% of the two proteins were present in a complex following elution from the second column.

Is this unstable complex composed of one copy of BC plus two copies of BCCP or a dimer of BC plus four copies of BCCP? Because BC is known to be dimeric (2, 5, 6, 14), the latter possibility seemed much more likely. To approach this question we analyzed the purified complex on non-denaturing gels with purified BC as a standard (Fig. 4). We found that the BC-BCCP complex had a slightly slower migration rate than BC. Because BC is dimeric, these results are consistent with a complex composed of two BC monomers and four BCCP monomers. It should be noted that, given the extra mass imparted to the BC dimer by binding of four BCCP molecules, one might have expected a somewhat slower migration rate for the complex. However, the effects of BCCP mass are offset by the greater negative charge of the complex, which would increase its mobility relative to BC. BC has a calculated isoelectric point of 6.95, whereas the values for BCCP and the 1:2 BC-BCCP complex are 4.5 and 5.4, respectively. Hence the relative electrophoretic migration rates we observed seem reasonable. Indeed, if the complex consisted of a monomer of BC plus two BCCP molecules this complex would be of lower molecular mass than the BC dimer (83 versus 98.8 kDa) and more acidic. Both properties should result in a species expected to migrate faster than the BC dimer. Because the complex migrates more slowly than the BC dimer, we conclude that the complex consists of a BC dimer plus four BCCP molecules, rather than the



FIG. 4. Analysis of the purified complex by native PAGE. BC and BCCP were co-expressed in *E. coli* strain BL21(DE3)pLysS carrying pER27 pCY216 and purified through a nickel chelate column, followed by elution from a monomeric avidin column as described under "Experimental Procedures." Hexahistidine-tagged BC protein was expressed in *E. coli* strain BL21(DE3)pLysS carrying pGLW2 and purified as previously described (32). Hexahistidine-tagged BC (lane 3) and the BC-BCCP complex (lane 4) were analyzed by 7% native PAGE, followed by Coomassie Blue staining. Lane 1 is a mixture of bovine serum albumin (67 kDa), and lane 2 is ovalbumin (43 kDa). The faint slowly migrating bands in lanes 1 and 2 represent the dimeric forms of bovine serum albumin and ovalbumin.

2BC·2BCCP complex previously assumed. The use of two affinity columns avoids the complication of an uncomplexed subunit binding to a column and thereby skewing the ratio. We used the tetrameric avidin matrix rather than the monomeric avidin matrix for the second column, because the tetrameric avidin bound BCCP much more rapidly than the monomeric species and the dissociation of the complex was thereby minimized. Unfortunately, the denaturing conditions required for elution from the tetrameric avidin matrix precluded use of the columns in the opposite order.

The Interaction Domain of BCCP—BCCP has two regions of known function. The C-terminal half of the protein is the tightly folded and highly conserved biotin domain for which both x-ray and 2D NMR structures are available (24–26). Upstream of the biotin domain is a linker region of about 42 residues with over half of residues being proline or alanine, which can be functionally replaced with a known linker region from an unrelated enzyme (18). Because an 87-residue biotin domain protein shows no sign of aggregation (11, 21) or of binding to BC (11, 21) and the linker region can be replaced with a foreign sequence (18), it seems that the remaining 30 or so N-terminal residues of BCCP must be responsible for interaction with BC and perhaps with the other ACC subunits. Consistent with this premise James and Cronan² have found that a high level of expression of the N-terminal half of BCCP is very toxic to *E. coli*. To directly test the role of the N-terminal region in BC·BCCP interaction we constructed a series of four amino acid residue deletions within this region and assayed the ability of the internally deleted proteins to interact with BC.

Nine sequential four-residue deletion mutants of BCCP, $\Delta 1$ – $\Delta 9$ (Fig. 5A), were constructed from plasmid pER2, which carries a genomic DNA fragment encoding BCCP and BC in-

serted into vector pQE60 (3.6 kb). This subcloning was done to obtain a small template plasmid suitable for PCR amplification. Plasmid pER2 was amplified with *Pfu* DNA polymerase using specific primers designed to introduce 12-bp deletions sequentially through the N-terminal coding sequence. All of the BCCP deletion mutant constructs gave high levels of BC and BCCP expression except the $\Delta 1$ and $\Delta 2$ constructs, which showed low level expression that was difficult to detect with Coomassie Blue staining. However, expression of BC and BCCP from $\Delta 1$ and $\Delta 2$ was confirmed by L-[³⁵S]methionine labeling (done as described above) after subcloning into pET-14b (data not shown). To assay interaction of BC and BCCP we used an avidin column to trap BCCP. We then eluted the specifically bound proteins and used SDS gel electrophoresis to assay for retention of BC through its interaction with BC. With the exception of the $\Delta 7$ and $\Delta 9$ constructs all of the BCCP deletion mutants failed to form a complex with BC as shown by the lack of BC in the column eluates. These data clearly demonstrate that an N-terminal domain of about 30 residues is required for BC-BCCP complex formation.

The most straightforward assembly pathway for the BC-BCCP complex would be for each of the subunit proteins to form homodimers that would then assemble into the final complex. Therefore, it seemed possible that only dimers of BCCP might bind BC and thus those mutant BCCPs unable to dimerize would fail to form a BC-BCCP complex. To test this possibility we expressed two of the mutant proteins and the wild type BCCP from a plasmid that lacked *accC* sequences (and thus encoded only BCCP) in the presence of [³H]biotin. The [³H]biotin-labeled BCCP species were then purified by elution from a monomer avidin column and analyzed by gel exclusion chromatography. The wild type BCCP migrated with an apparent molecular mass of 54 kDa, whereas the $\Delta 7$ protein (which also forms a BC-BCCP complex, Fig. 5) had a similar elution pattern except that a lower molecular mass species of 29 kDa was also present (Fig. 6). In contrast the $\Delta 4$ BCCP was present mainly as the smaller species, although about one-fourth of the protein was in the larger 54-kDa species. BCCP is known to be a protein of very unusual hydrodynamic properties that presumably result from its atypical shape plus the flexibility of the long linker region. Indeed based on hydrodynamic measurements (1, 13) BCCP was thought to be considerably larger than its true mass of 16.7 kDa, which was later established by analysis of the *accB* gene (17) and by mass spectral analysis (11). Therefore, we believe that the apparent 29- and 54-kDa forms are the monomeric and dimeric forms of BCCP, respectively. At first glance it seemed as though the inability of $\Delta 4$ BCCP to form a BC-BCCP complex could be attributed to a defect in dimerization. However, because about one-fourth of the protein was dimeric some BC-BCCP complex should have been detectable in the experiment of Fig. 5. Because no BC-BCCP complex was seen, it seemed that $\Delta 4$ BCCP had defects in both dimerization and in interaction with BC that were at least partially independent of one another. This result suggests that delineation of these protein interactions will require mutations more subtle than the deletions we have constructed.

The BC-BCCP Complex Can Be Biotinylated in Vitro—It seems possible that biotinylation of BCCP could occur either before or after assembly of the protein into the BC-BCCP complex. Purified apo-BCCP is a substrate for biotinylation by BirA *in vitro* (11), but the BC-BCCP complex had not been tested as a substrate. We tested the BC-BCCP complex as a BirA substrate by expressing the altered *accBC* operon that encoded the hexahistidine-tagged BC in medium lacking biotin supplementation such that an under-biotinylated BC-BCCP complex

² E. S. James and J. E. Cronan, unpublished data.

FIG. 5. Effect of deletions within the BCCP N terminus on formation of the BC-BCCP complex. A, the deleted residues are indicated by empty spaces. B, BC and BCCP were co-expressed in *E. coli* strain TOP10F' carrying each construct plus pCY216 and purified through a NeutrAvidin column as described under "Experimental Procedures." The eluates from each of the columns were analyzed by 12% SDS-PAGE followed by Coomassie Blue staining.

	N-Terminal sequence	Complex formation?
WT	MDIRKIKKLIELVEESGISELEISEGEEESVRISRAAP	Yes
Δ1	M IKKLIELVEESGISELEISEGEEESVRISRAAP	No
Δ2	MDIRK IELVEESGISELEISEGEEESVRISRAAP	No
Δ3	MDIRKIKKL EESGISELEISEGEEESVRISRAAP	No
Δ4	MDIRKIKKLIELV ISELEISEGEEESVRISRAAP	No
Δ5	MDIRKIKKLIELVEESG EISEGEEESVRISRAAP	No
Δ6	MDIRKIKKLIELVEESGISEL GEESVRISRAAP	No
Δ7	MDIRKIKKLIELVEESGISELEISE VRISRAAP	Yes
Δ8	MDIRKIKKLIELVEESGISELEISEGEEES RAAP	No
Δ9	MDIRKIKKLIELVEESGISELEISEGEEESVRIS	Yes

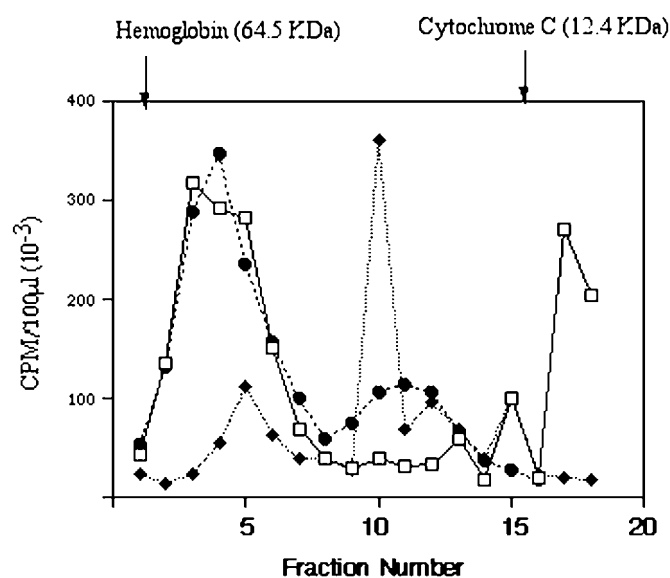
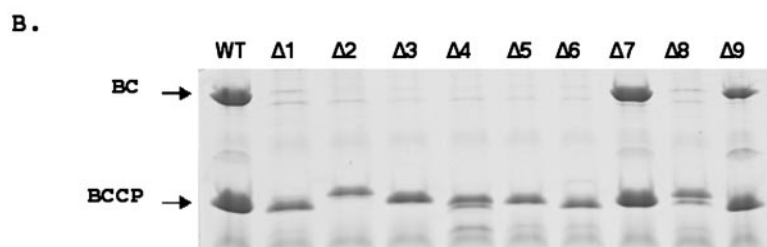


FIG. 6. Molecular forms of BCCP species. [³H]Biotin-labeled BCCP species were purified, mixed with hemoglobin and cytochrome *c* as internal standards, and then analyzed by gel filtration on a G-75 Sephadex column (see "Experimental Procedures"). Symbols: □, wild type BCCP; ■, Δ7 BCCP; and ◆, Δ4 BCCP.

would accumulate. This complex was purified on a nickel chelate column and then biotinylated *in vitro* by treatment with purified BirA plus ATP and biotin. A marked increase in protein-bound biotin was observed by Western blotting using a streptavidin conjugate (data not shown). However, it remained possible that the BC-BCCP complex had dissociated following elution from the nickel chelate column and that the biotinylation substrate was not the intact complex, but free BCCP that had dissociated from the complex. To test this possibility we passed the BirA-treated proteins over an avidin column to determine if the BCCP was still complexed to BC. This analysis showed a marked increase in the amount of BC bound by the avidin column upon treatment with BirA, ATP, and biotin (Fig. 7). We conclude that the intact BC-BCCP complex was a substrate for BirA-catalyzed biotinylation. The *Propionibacterium shermanii* transcarboxylase and its subcomplexes have also been reported to be biotinylation substrates *in vitro* (27).

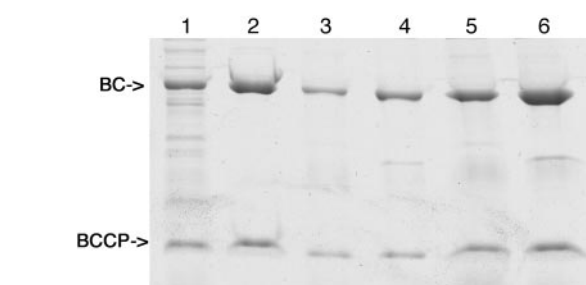


FIG. 7. Biotinylation of the BC-BCCP complex *in vitro*. Under-biotinylated BC-BCCP was obtained, purified, and treated with *E. coli* BirA protein, ATP, and biotin as described under "Experimental Procedures." The reaction mixtures were then assayed for the BC-BCCP complex by NeutrAvidin binding (Fig. 5). Lane 1, crude extract; lane 2, nickel chelate-purified complex; lanes 3 and 5 are samples of the complex that were not biotinylated *in vitro*; lanes 4 and 6 are samples of the complex that had been biotinylated *in vitro*. The amounts of complex loaded in lanes 3 and 4 were identical as were those of lanes 4 and 6. The amounts loaded in lanes 5 and 6 were 4-fold greater than those of lanes 3 and 4.

DISCUSSION

Our finding that the molar ratio of BC to BCCP in the BC-BCCP complex isolated by the double chromatography method is 1:2 was unexpected in view of the prior report on the *P. citronellolis* acetyl-CoA carboxylase complex, which gave a 1:1 BC to BCCP ratio (9). However, this estimate was based on Coomassie Blue staining, which varies significantly among proteins with acidic proteins such as BCCP (pI 4.5) binding the least amount of stain per protein mass (28). As expected we find that Coomassie Blue staining of BCCP is roughly 5-fold weaker than that seen with typical proteins (data not shown). We expect that, given the very high degrees of amino acid sequence similarity between the ACC subunits of *E. coli* and pseudomonads, the structures of the ACC complexes are the same and therefore postulate that Fall (9) underestimated the relative amount of BCCP due to relatively poor staining of the protein. Radioactive methionine-labeled crude extracts contain BC and BCCP in a 1:2 molar ratio, and thus the two proteins are made in the same ratio in which they are assembled into the complex. This makes good physiological sense, because neither protein has an enzymatic function in the absence of the other. It is not unusual to find that the relative levels of proteins encoded within an operon are directly related to the

proximity of the encoding gene to the promoter as we observed for the *accBC* operon. Indeed this was first seen in the *E. coli* lactose operon (29). The need for more BCCP than BC may provide an explanation for the very highly (but not absolutely) conserved *accBC* gene arrangement found in the genomes of very diverse bacteria (30). It should be noted that the transcarboxylase of *P. shermanii*, another bacterial biotin-dependent enzyme, consists of a stable complex of three proteins that have functions analogous to the ACC subunits of *E. coli* (8, 31). The ratio of the 12 S biotin carboxylase subunit to the biotinylated 1.3 S subunit is 1:2, the same as that we observed for the *E. coli* BC·BCCP complex.

The finding that the BC·BCCP complex has two BCCP molecules per BC molecule removes the conflict between two models that have been put forth for the action of BC and (by extension) of ACC (19, 32). In prior work it was found that the two subunits of BC communicate in that mutations that inactivate one active site of a dimer inactivate the other active site even though the active sites are located far from the dimer interface and from one another (32). This dominant negative effect led to the following model. The two subunits of BC may be unable to catalyze the reaction simultaneously. Instead, perhaps the two subunits must alternate catalytic reactions, such that while one subunit is binding substrate and undergoing catalysis, the other subunit is releasing product. If one of the subunits has a mutation resulting in a significant decrease in catalytic rate, this would also lead to a decrease in the catalytic rate of the wild-type subunit, because the catalytic sequences of the two subunits would be mechanistically linked in a fixed cycle. Given one BCCP per BC this model postulated that the two BCCP molecules of the BC·BCCP must be well separated from one another during catalysis, because each would be interacting with a different BC active site. A second model emerged from work showing that co-expression of two mutant BCCP proteins, each of which was non-functional when expressed alone, could restore BCCP function (19). The most straightforward explanation for these observations was that at least two biotinoyl domains interact during the ACC reaction implying that the domains are located close to one another. Given a 1:1 BC·BCCP ratio either the first or second model was surely incorrect. However, given two BCCP molecules per BC, the models no longer conflict and allow us to postulate a new composite model. In this model a pair of BCCP molecules would interact with each BC active site. The postulated interaction within each BCCP pair can explain the stabilization of one mutant protein molecule by another, and the two BCCP pairs could reciprocate in the mechanistically linked fixed cyclic reaction postulated earlier and thus explain the dominant negative effects on BC activity.

The structural interactions that form the BC·BCCP complex are unknown but clearly involve the N-terminal 24–32 residues of BCCP. An interesting finding is that the BCCP of *Bacillus subtilis* can functionally replace that of *E. coli* as demonstrated by genetic complementation of an *E. coli* BCCP mutant (33). This complementation indicates that this foreign BCCP interacts properly with the *E. coli* BC and carboxyltransferase proteins. However, alignments of the N-terminal se-

quences of the *E. coli* and *B. subtilis* BCCPs show minimal sequence homology, only 9 of the 36 N-terminal residues are identical and only a minority of the remaining residues are similar. In light of these differences it is surprising that the *B. subtilis* BCCP is fully functional in *E. coli* upon expression at the same level as that of the *E. coli* protein transcribed from the chromosomal *accB* gene.² The major conserved features of the N termini of these two BCCPs and that of *P. aeruginosa* (which like *B. subtilis* BCCP can functionally replace the *E. coli* protein *in vivo* (34)) are a conserved spacing of hydrophobic aliphatic residues. Although the conserved pattern of aliphatic residues is reminiscent of structures such as leucine zippers and coiled-coils, the pattern fits no reported tertiary structure motif and threading programs produce no convincingly homologous structures.

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