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## Complex formation and regulation of Escherichia coli acetyl-CoA carboxylase

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COMPLEX FORMATION AND REGULATION OF *ESCHERICHIA COLI* ACETYL-COA  
CARBOXYLASE

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Biological Sciences

by  
Tyler Craig Broussard  
B.S., Louisiana State University, 2006  
May 2013

To Ashley:

I forged and sharpened the blade, but you did the quenching and tempering.

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## ABSTRACT

Acetyl-CoA carboxylase is a biotin-dependent, multifunctional enzyme that catalyzes the first committed step in fatty acid synthesis. The *Escherichia coli* enzyme is composed of a homodimeric biotin carboxylase, biotinylated biotin carboxyl carrier protein (BCCP), and an  $\alpha_2\beta_2$  heterotetrameric carboxyltransferase. Catalysis by acetyl-CoA carboxylase proceeds via two half-reactions. In the first half-reaction, biotin carboxylase catalyzes the ATP-dependent carboxylation of biotin, which is covalently attached to BCCP, to form carboxybiotin. In the second half-reaction, carboxyltransferase transfers the carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA. All biotin-dependent carboxylases are proposed to have a two-site ping-pong mechanism where the carboxylase and transferase activities are separate and do not interact. This posits two hypotheses: either biotin carboxylase and BCCP undergo the first half-reaction, BCCP dissociates, and then BCCP interacts with carboxyltransferase to complete the second-half reaction, or all three components form a macromolecular enzyme complex. To determine which hypothesis is correct, a steady-state enzyme kinetic analysis of *E. coli* acetyl-CoA carboxylase was carried out. The results indicated the two active sites of acetyl-CoA carboxylase interact. Both *in vitro* and *in vivo* pull-down assays demonstrated that the three components of *E. coli* acetyl-CoA carboxylase form a multimeric complex and that complex formation is unaffected by acetyl-CoA, AMPPNP, and mRNA coding for carboxyltransferase.

The second study focuses on the crystallization of part of the acetyl-CoA carboxylase complex. To date, the three-dimensional structures of the individual subunits, except the N-terminal domain of BCCP, have been solved; however, the structural basis for how BCCP reacts with biotin carboxylase or carboxyltransferase is unknown. Therefore, we report here the first crystal structure of *E. coli* BCCP complexed with biotin carboxylase to a resolution of 2.49 Å.



The protein-protein complex shows unique quaternary structure and two distinct interfaces for each BCCP monomer. The structure supports a model by which biotin protein ligase can biotinylate the complex. The BCCP binding sites are unique compared to phylogenetically related biotin-dependent carboxylases and therefore, provide novel targets for developing antibiotics against bacterial acetyl-CoA carboxylase. Taken together, these findings provide structural and functional insight into the regulation and complex formation of *E. coli* acetyl-CoA carboxylase.

## CHAPTER 1 INTRODUCTION

### Biotin

#### Background

Vitamin H or B<sub>7</sub>, commonly known as biotin, is required for life by animals, plants and bacteria. Many prokaryotes and plants synthesize their own biotin while mammals obtain this nutrient through intestinal uptake after synthesis by normal flora of the gut or consumption of foods such as egg yolk, organ meats, nuts and some vegetables. The first reference to biotin in the literature is from Wildiers (1901), who found that a substance he termed “bios”, later determined to be biotin and other nutrients, was necessary for yeast growth. Biotin was isolated from egg yolk (Kogl and Tonnis, 1936) and subsequently from beef liver (Vitamin H) and *Rhizobium sp.* (cofactor-R), which were all found to be the same compound (Du Vigneaud *et al.*, 1941; Gyorgy *et al.*, 1940; Melville *et al.*, 1940). Additionally, Bateman (1916) found that egg whites caused gastrointestinal problems and malabsorption of protein in dogs, rats, rabbits and humans. Shortly after, Eakin *et al.* (1941) identified avidin as the protein in egg whites that causes a dietary deficiency of biotin, due to binding biotin with a  $K_d = 10^{-15}$  M (Green, 1990). The next step was determination of the structure of biotin.

#### Structure

Biotin is a bicyclic compound containing a ureido ring fused *cis* to a tetrahydrothiophene ring (Figure 1.1). The structure of biotin was elucidated by Du Vigneaud and Melville (1942) and the first *de novo* synthesis was described by Harris *et al.* (1944). The molecule has a molecular weight of 244.31 and of the eight stereoisomers that exist, only D-(+)-biotin is found and active in nature (Streit and Entcheva, 2003). A valeric acid side chain is attached to one of

the three contiguous chiral carbons in the thiophane ring. Covalent attachment to biological molecules, such as proteins and nucleic acids, occurs via the valeric acid side chain (Hermanson, 2008) and allows for convenient detection of these biotinylated compounds using avidin (Green, 1990). The discovery of the unique interaction between avidin and biotin has led to the development of several techniques in biotechnology for the detection of biotinylated proteins and nucleic acids (Diamandis and Christopoulos, 1991). In contrast to the beneficial biotechnical uses of biotin, nature synthesizes biotin for a different reason.

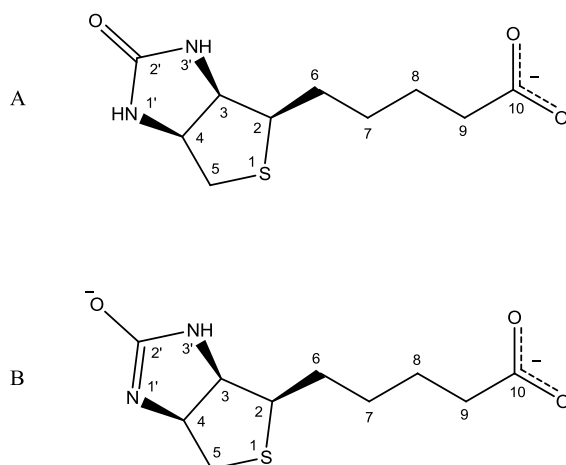


Figure 1.1 Structure of biotin. A) D-(+)-biotin. B) The imidate form of A.

## Biosynthesis

The expression of several genes is required for biotin biosynthesis. *Escherichia coli* expresses the biotin biosynthetic genes in the *bioABFCD*<sup>1</sup> operon as well as the *bioH* gene located outside of this operon (Marquet *et al.*, 2001; Otsuka and Abelson, 1978). This is a bi-directional operon in that the *bioB*, *bioF*, *bioC*, and *bioD* genes are rightward transcribed and the *bioA* gene is leftward transcribed (Streit and Entcheva, 2003). Biotin biosynthesis begins with

<sup>1</sup>Throughout the text, genes will be represented in italics and proteins will be represented as a plain text version of the gene or by their common name. For example, *bioB* is the gene encoding the protein BioB or biotin synthase.

the conversion of either malonyl-CoA or malonyl-ACP into malonyl-CoA (or ACP) methyl ester via the S-adenosyl-L-methionine (SAM)-dependent methylation carried out by BioC (Lin *et al.*, 2010) (Figure 1.2). This molecule then replaces acetyl-CoA in fatty acid synthase and undergoes two cycles of condensation, reduction, dehydration and reduction to produce a C7, pimeloyl-ACP methyl ester, which is then hydrolyzed by BioH to yield pimeloyl-ACP and methanol (Lin *et al.*, 2010). Next, the condensation of pimeloyl-ACP and L-ala produces 7-keto-8-aminopelargonic acid (KAPA) and the reaction is catalyzed by KAPA synthase (BioF) (Streit and Entcheva, 2003). SAM then acts as an amino donor to KAPA to produce 7,8-diaminopelargonic acid (DAPA) in a reaction catalyzed by DAPA synthase (BioA) (Breen *et al.*, 2003). Dethiobiotin synthetase (BioD) is responsible for the ATP-dependent closure of the ureido ring, which creates dethiobiotin from DAPA (Marquet *et al.*, 2001). Finally, biotin synthase (BioB) uses two equivalents of SAM to close the tetrahydrothiophene ring creating biotin (Figure 1.2) (Fugate and Jarrett, 2012). Currently, the mechanism of biotin synthase is uncertain, but the sulfur atom is thought to be donated from an  $[2Fe-2S]^{2+}$  cluster (Fugate and Jarrett, 2012). Biotin can now be attached to the biotin carboxyl carrier protein (BCCP) of biotin-dependent enzymes to act as a carboxyl carrier for carboxylation, decarboxylation, and transcarboxylation reactions in various organisms.

## **Biotin-dependent Enzymes**

### Background

Biotin-dependent enzymes use the biotin prosthetic group to transfer or remove carboxyl groups. They all proceed by two half-reactions that involve the carboxylation and then

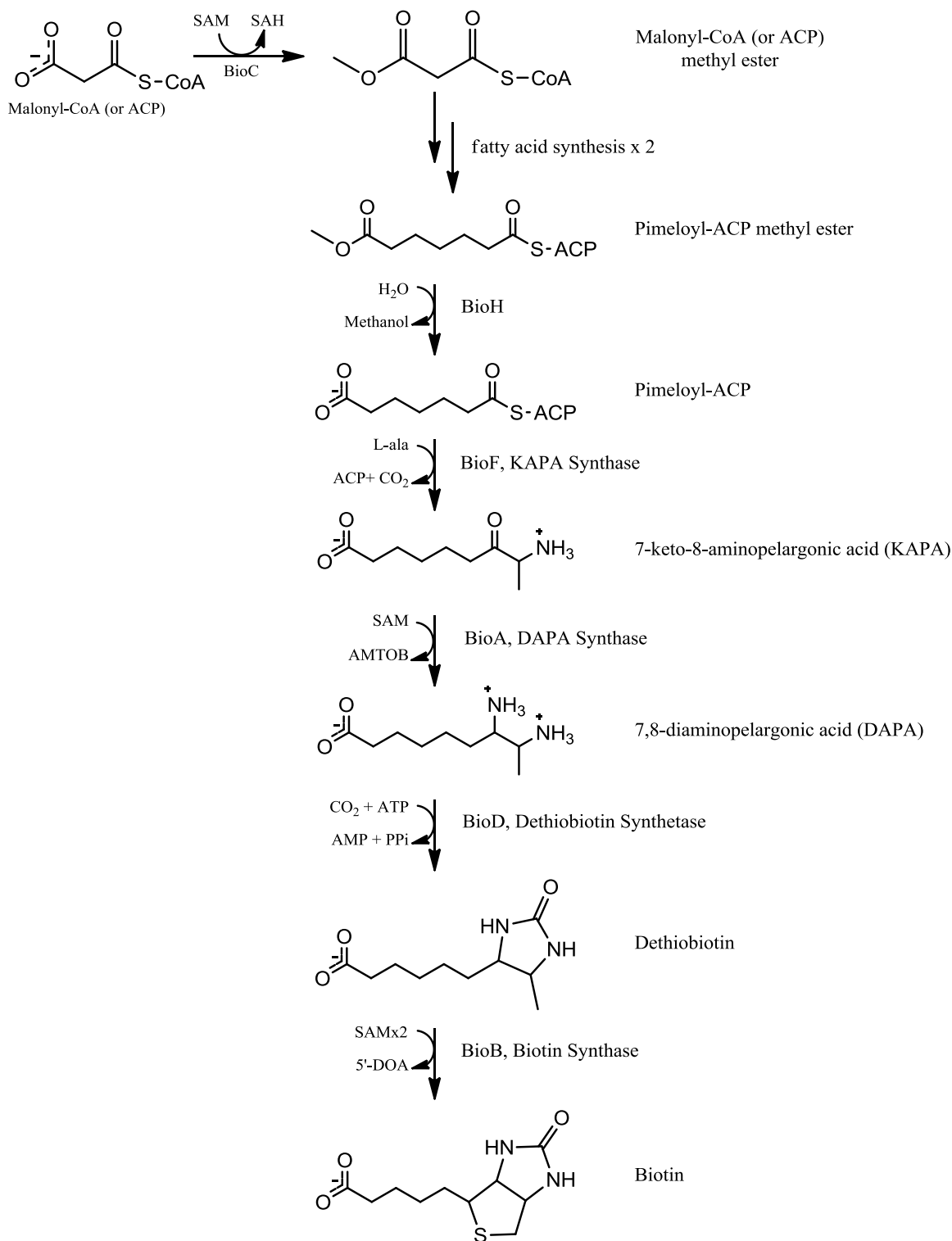


Figure 1.2 Biosynthesis of biotin in *E. coli*. Abbreviations: SAH, S-adenosylhomocysteine; AMTOB, S-adenosyl-2-oxo-4-methylthiobutyric acid; 5'-DOA, 5'-deoxyadenosine.

decarboxylation of biotin at its 1'N and have been designated as having non-classical or two-site ping-pong mechanisms (Wood and Barden, 1977). These multifunctional enzymes are involved in a range of metabolic processes and are divided into three classes based on their use of the carboxyl group in carboxylation (Class I), decarboxylation (Class II), or transcarboxylation (Class III) (Figure 1.3).

#### Class I: The Carboxylases

The carboxylases represent the largest class of biotin-dependent enzymes and the only one of the three found in mammals. These enzymes are named after the acceptor molecule they carboxylate and include pyruvate carboxylase, propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, acetyl-CoA carboxylase, geranyl-CoA carboxylase and urea amidolyase, which produce oxaloacetate, methylmalonyl-CoA, 3-methylglutaconyl-CoA, malonyl-CoA,  $\gamma$ -carboxygeranyl-CoA and ammonia, respectively (Lombard and Moreira, 2011). They contain three subunits or domains depending on whether they are synthesized separately or on the same polypeptide chain: biotin carboxylase (BC), biotin carboxyl carrier protein and carboxyltransferase (CT). In the first half-reaction, BC catalyzes the ATP-dependent carboxylation of biotin that is covalently attached to the BCCP (Figure 1.3 A). The carboxyl group is derived from bicarbonate in all of the carboxylases (Rubio, 1986). The second half-reaction, catalyzed by carboxyltransferase (CT), involves the transfer of the carboxyl group from biotin to an acceptor molecule. The carboxylases are important for gluconeogenesis, the catabolism of odd-chain fatty acids and branched chain amino acids, fatty acid synthesis, isoprenoid catabolism, and the utilization of urea as a nitrogen source.

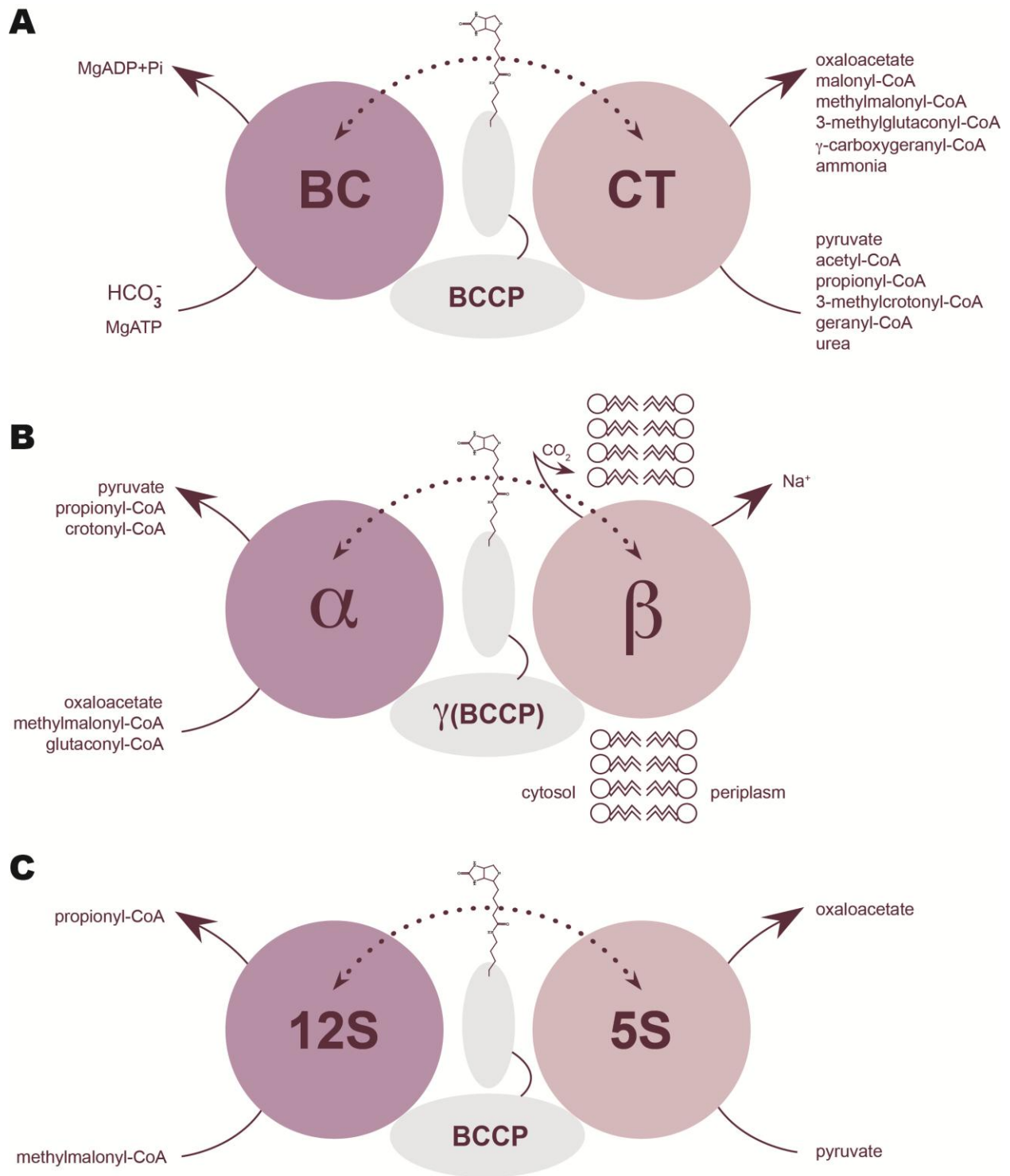


Figure 1.3 Cartoon depiction of the biotin-dependent enzyme reactions and major subunits. A) carboxylases. B) decarboxylases. C) transcarboxylase.

## Class II: The Decarboxylases

The three biotin-dependent decarboxylases are membrane bound and found in anaerobic bacteria and archaea. These enzymes are named for the substrate they decarboxylate and include oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, and glutaconyl-CoA decarboxylase. They consist of three main subunits: an  $\alpha$ -subunit that has the carboxyltransferase activity, a  $\beta$ -subunit that catalyzes the decarboxylation and sodium transport, and a  $\gamma$ -subunit, which is usually the biotin carrying protein, except in oxaloacetate decarboxylase where it is attached to the flexible C-terminus of the  $\alpha$ -subunit (Buckel, 2001; Jitrapakdee and Wallace, 2003). In the first half-reaction, the  $\alpha$ -subunit catalyzes the transfer of a carboxyl group from the substrate to biotin to form carboxybiotin. In the second half-reaction, biotin is decarboxylated, the carbon dioxide is released to the cytosol, a proton is transported into the cell, and one to two sodium ions are transported across the cytoplasmic membrane by the  $\beta$ -subunit (Figure 1.3 B) (Balsera *et al.*, 2011). The transport of sodium to the periplasm generates an electrochemical gradient that can then be used for ATP synthesis (Hase *et al.*, 2001), solute uptake (Pos and Dimroth, 1996), and flagellar motion (Berg, 2003; Kojima *et al.*, 1999).

## Class III: Transcarboxylase

Currently, only one enzyme falls into class III, transcarboxylase from *Propionibacterium shermanii*. Like the other classes, transcarboxylase is made up of three main subunits: six 12S subunits form a hexameric core, twelve 1.3S subunits carry the biotin prosthetic group and are attached to opposite ends of the 12S subunits, and three homodimers of the 5S subunit cap each end of the complex (Carey *et al.*, 2004). In the first half-reaction catalyzed by the 12S subunit, carboxybiotin is generated by decarboxylation of methylmalonyl-CoA and subsequent formation



of propionyl-CoA (Figure 1.3 C). In the second half-reaction, the 5S subunit transfers the carboxyl group from carboxybiotin to pyruvate to produce oxaloacetate (Jitrapakdee and Wallace, 2003). This transcarboxylase reaction is important for propionate metabolism.

## **Biotin Protein Ligase**

### Background

Biotin protein ligase (BPL), also known as BirA as well as holocarboxylase synthetase, is the enzyme responsible for post-translational attachment of biotin to the various biotin-dependent enzymes. It was originally thought that each biotin-dependent enzyme had its own ligase. However, gene sequence analysis and studies of biotinylation of apocarboxylases by bacterial and eukaryotic BPLs have shown that each organism contains one BPL that can biotinylate multiple biotin-dependent enzymes (Chapman-Smith and Cronan, 1999b; Leondelrio *et al.*, 1995; McAllister and Coon, 1966). The enzyme undergoes two half-reactions that covalently link biotin to a specific lysine residue on the carrier protein of each biotin-dependent enzyme. First, BPL catalyzes the formation of biotinoyl-5'-AMP from biotin and ATP. Second, biotin is transferred from the biotinoyl-5'-AMP adduct to form an amide bond between the  $\epsilon$ -amino group of lysine and the carboxylic acid on the valeric acid side chain of biotin (Figure 1.4) (McAllister and Coon, 1966). The structure of these enzymes have been shown to be similar to tRNA synthetases, which catalyze a very similar type of reaction (Leatherbarrow *et al.*, 1985; Safro and Mosyak, 1995).

### Regulation of Biotin Protein Ligase

BPL was found to be a bi-functional enzyme when it copurified with the DNA of the *bio* operon (Barker and Campbell, 1981). In *E. coli*, BPL is a 35.5 kDa protein that has an N-

terminal domain with a DNA binding helix-turn-helix motif (Wilson *et al.*, 1992). In the absence of BCCP, the sole biotinylated protein in *E. coli*, the co-repressor biotinoyl-5'-AMP, remains tightly bound to BPL (Prakash and Eisenberg, 1979). The buildup of BPL-biotinoyl-5'-

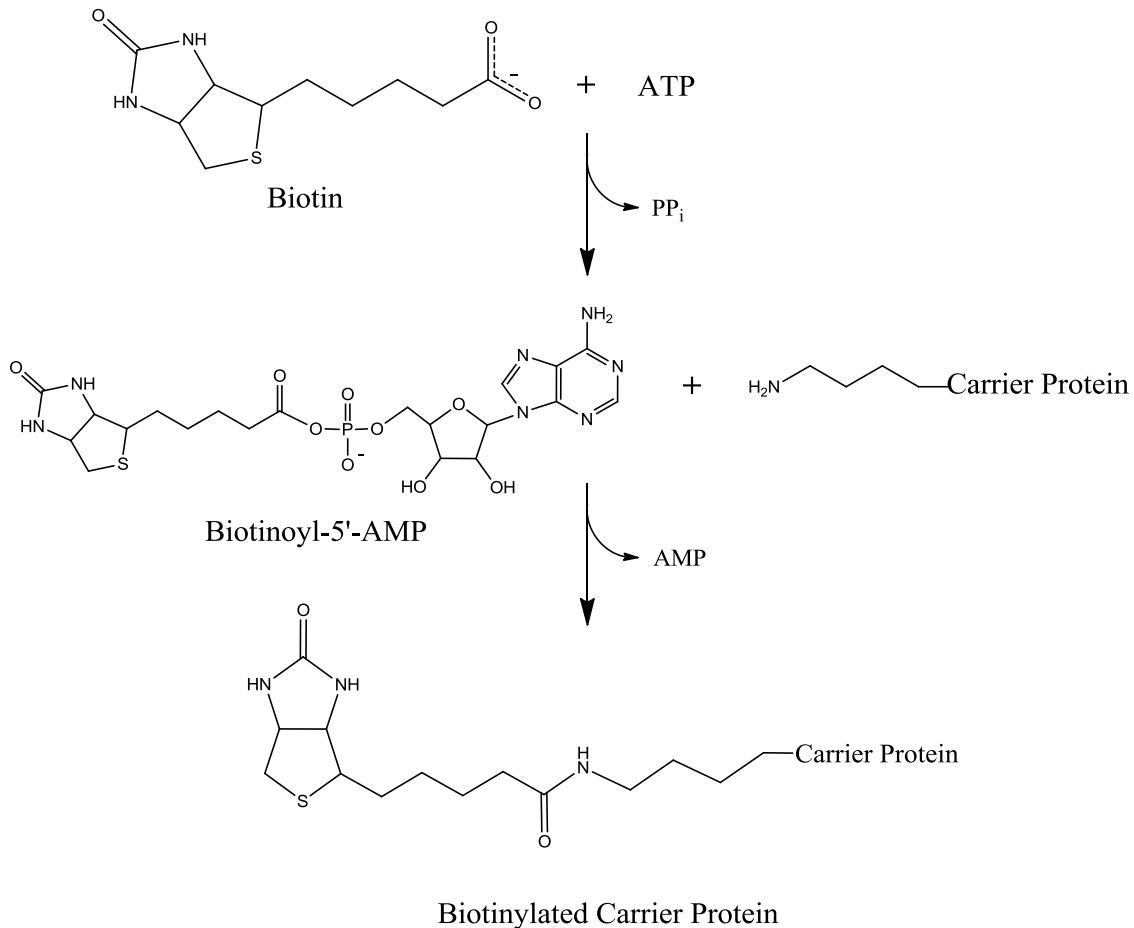


Figure 1.4 Biotin attachment to biotin-dependent carrier proteins.

AMP complex causes dimerization, which allows binding to the 40 bp biotin operator, *bioO* (Streaker and Beckett, 1998). This down-regulates transcription of the biotin biosynthetic genes (Prakash and Eisenberg, 1978). Sequence analysis of other microorganisms show that the N-terminal BPL domain and the operon sequence is highly conserved, suggesting biotin synthesis is regulated in a similar manner in other organisms that synthesize biotin (Rodionov *et al.*, 2002).

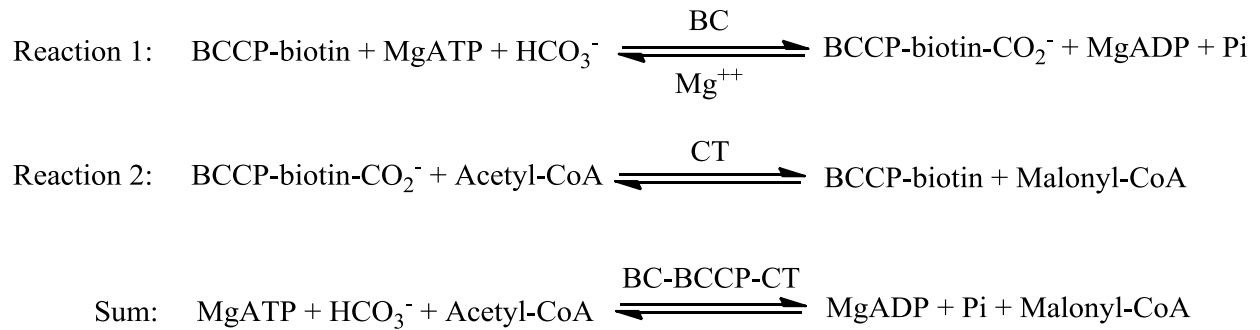
In contrast, gene sequence analysis suggests there are no sequences indicative of DNA binding in the BPL of organisms that do not synthesize their own biotin (Chapman-Smith and Cronan, 1999b). Finally, synthesis of apoBCCP causes derepression of the biotin operon because conversion of biotinoyl-5'-AMP to biotinylated BCCP and AMP causes BPL to lose its DNA binding function (Cronan, 1988). Ultimately, this system works to sense the biotin requirements of the cell and express biotin as needed to biotinylate acetyl-CoA carboxylase.

## **Acetyl-CoA Carboxylase**

### Background

Acetyl-CoA carboxylase (ACC) from *E. coli* is the focus of the research in this dissertation and thus will be described in detail. Relevant aspects of its structure and function will be compared to other carboxylases and its significance as an antibacterial target will also be considered.

Acetyl-CoA carboxylase was discovered when Wakil *et al.* (1958) showed that avidin inhibited the conversion of acetyl-CoA to palmitate in enzymatic fractions from avian livers. Acetyl-CoA carboxylase catalyzes the first committed step in the biosynthesis of long chain fatty acids in all plants, animals, and bacteria (Wakil *et al.*, 1983). All acetyl-CoA carboxylases catalyze the conversion of acetyl-CoA to malonyl-CoA by two half-reactions. The first half-reaction, catalyzed by biotin carboxylase, is the ATP-dependent carboxylation of the vitamin biotin, which is covalently attached to the carrier protein (Reaction 1 in Scheme 1.1) (Alberts *et al.*, 1969). In the second half-reaction, carboxyltransferase transfers the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA (Reaction 2 in Scheme 1.1) (Alberts *et al.*, 1971; Guchhait *et al.*, 1971).



Scheme 1.1 Acetyl-CoA carboxylase half-reactions and sum.

### Subunit Arrangement

The subunit architecture of acetyl-CoA carboxylase from bacteria to humans has been divided into either type I fatty acid synthesis (FAS I) or type II fatty acid synthesis (FAS II) (Figure 1.5) (Rock and Cronan, 1996). In FAS I, proteins are expressed as multiple domains on a single polypeptide chain. In contrast, FAS II proteins are expressed as dissociated parts that interact to catalyze the reaction (Rock and Cronan, 1996).

Acetyl-CoA carboxylase from *E. coli* is a model of a FAS II enzyme. It is made up of four separately expressed proteins that form its three subunits: a homodimeric biotin carboxylase, a biotinylated biotin carboxyl carrier protein, and an  $\alpha_2\beta_2$  heterotetrameric carboxyltransferase (Figure 1.5). The genes for BCCP (*accB*) and BC (*accC*) are cotranscribed from an *accBC* minioperon located at min 73.35 on the *E. coli* chromosome (James and Cronan, 2004; Li and Cronan, 1992a). The genes encoding the  $\alpha$ -subunit (*accA*) and  $\beta$ -subunit (*accD*) of CT are located on opposite sides of the chromosome at min 4.5 and 52.4, respectively (Li and Cronan, 1992b). Most bacteria and plant chloroplasts contain a multisubunit ACC similar to the one observed in *E. coli* (Cronan and Waldrop, 2002).

In contrast to the subunit arrangement in *E. coli* acetyl-CoA carboxylase, *Mycobacterium sp.*, *Streptomyces sp.*, and *Myxococcus xanthus* transcribe a gene that encodes an  $\alpha$ -subunit di-domain polypeptide containing the carboxylase and carrier protein and a gene that encodes a  $\beta$ -subunit containing the carboxyltransferase as a di-domain polypeptide (Figure 1.5) (Kimura *et al.*, 2000). These enzymes form  $\alpha_6\beta_6$  quaternary structures and have been termed acyl-CoA

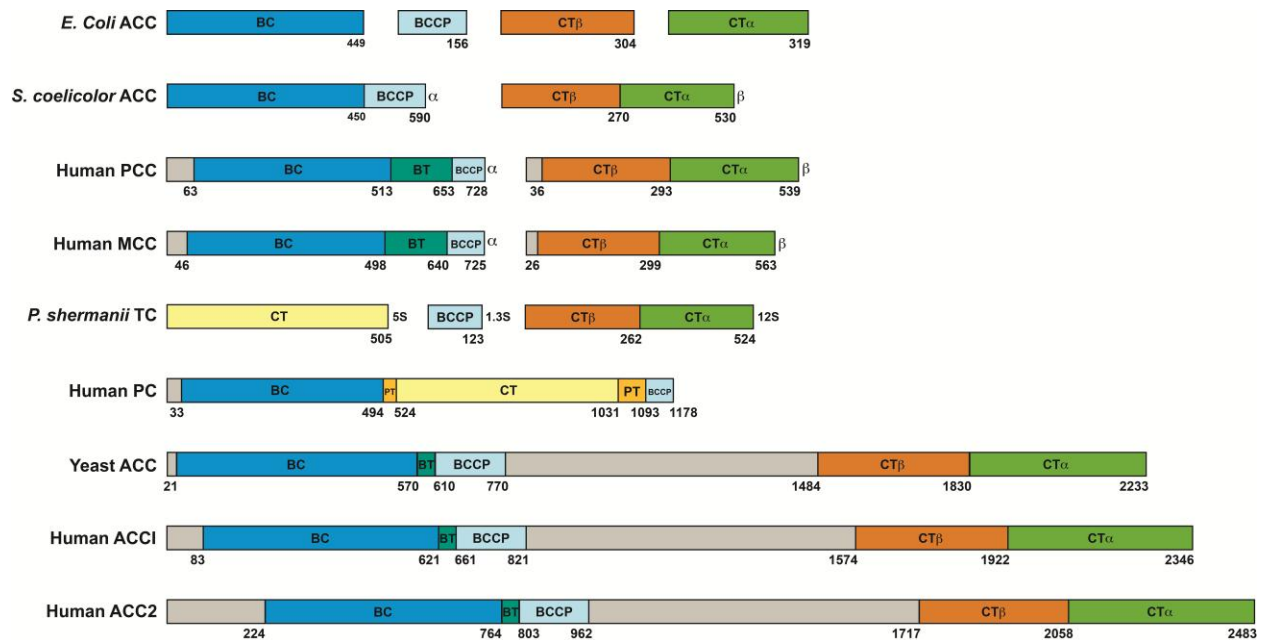


Figure 1.5 Domain architecture of acetyl-CoA carboxylase homologs. Domain architecture of various biotin-dependent enzymes are depicted as primary protein sequences indicated by colored boxes with residue numbers showing the length beneath. Domains and subunits that are similar are labeled and color coded to match. The organism the protein is expressed in proceeds the functional enzyme name: ACC (acetyl-CoA carboxylase), PCC (propionyl-CoA carboxylase), MCC (methylcrotonyl-CoA carboxylase), TC (transcarboxylase) or PC (pyruvate carboxylase). The PT (PC tetramerization) domain is shown in light orange and the BT domain is shown in teal and are involved in BC and CT domain or subunit interactions.

carboxylases because they can catalyze the carboxylation of several acyl-CoAs, such as acetyl-CoA, propionyl-CoA, and butyryl-CoA (Diacovich *et al.*, 2002). For instance, the *accA1* and *accA2* genes from *Streptomyces coelicolor* encode nearly identical  $\alpha$ -subunits that can interact

with either of the *accB* or *pccB* gene products (Rodriguez *et al.*, 2001; Rodriguez and Gramajo, 1999). Formation of an AccA and AccB complex yields an acyl-CoA carboxylase that was able to carboxylate acetyl-CoA, propionyl-CoA, and butyryl-CoA, whereas an AccA and PccB complex would not carboxylate acetyl-CoA (Diacovich *et al.*, 2002). Furthermore, *Mycobacterium tuberculosis* expresses three  $\alpha$ -subunits (*accA1-3*) and six  $\beta$ -subunits (*accD1-6*) (Gago *et al.*, 2006). The  $\beta$ -subunits have differing affinities for acetyl-CoA, propionyl-CoA and butyryl-CoA. Additionally, an  $\epsilon$ -subunit has been shown to be required for maximal catalytic activity of the  $\alpha$ - $\beta$ -subunit complex of *M. tuberculosis* (Gago *et al.*, 2006) while in *S. coelicolor*, an  $\epsilon$ - $\beta$  subcomplex is formed first, which is then able to interact with the  $\alpha$ -subunit and allow maximal activity (Diacovich *et al.*, 2002). Human propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase are also expressed as an  $\alpha$ -subunit containing the carboxylase and carrier protein and a  $\beta$ -subunit with the transferase function that forms an  $\alpha_6\beta_6$  dodecamer with a small domain incorporated into the  $\alpha$ -subunit that mediates binding between subunits (Figure 1.5) (Huang *et al.*, 2012; Huang *et al.*, 2010). These observations provide a convincing argument that the carboxylases from *M. tuberculosis*, *S. coelicolor* and *M. xanthus* are evolutionary precursors to the eukaryotic form of ACC because fusion of the genes encoding the  $\alpha$ - and  $\beta$ -subunits of these organisms ACCs could account for the arrangement observed in eukaryotes.

In animals, the three components of ACC form different domains on one polypeptide chain (Figure 1.5) (Tanabe *et al.*, 1975). Two isoforms of ACC expressed in humans. A 265 kDa isoform involved in fatty acid synthesis (ACC1) in the liver, adipose tissue and mammary glands and a 280 kDa isoform involved in the inhibition of fatty acid oxidation (ACC2) in the liver, heart, and skeletal muscle (Munday, 2002). These large multifunctional enzymes come together

to form dimeric quaternary structures in humans and tetramers in yeast (Brownsey *et al.*, 2006; Tong, 2005; Vagelos *et al.*, 1963).

## **Individual Subunits of *E. coli* Acetyl-CoA Carboxylase**

### Biotin Carboxylase

The structure of biotin carboxylase from *E. coli* solved by Waldrop *et al.* (1994) was the first structure of a biotin-dependent carboxylase. Each monomer of BC contains 449 amino acid residues that are divided into three domains: an N-terminal domain, a B-domain or ATP-grasp domain, and a C-terminal domain (Figure 1.6) (Waldrop *et al.*, 1994). The N-terminal domain, comprising residues Met1-Ile103, contains a  $\beta$ -pleated sheet with five parallel strands and a total of four  $\alpha$ -helices (Waldrop *et al.*, 1994). The ATP-grasp domain is made up of residues Val131-Tyr203 and has three strands of  $\beta$ -pleated sheet and two  $\alpha$ -helices (Thoden *et al.*, 2000). The C-terminal domain accounts for the largest part of BC spanning residues Arg208-Lys449. This domain contains an eight stranded antiparallel  $\beta$ -sheet, a smaller three stranded antiparallel  $\beta$ -sheet and seven  $\alpha$ -helices (Waldrop *et al.*, 1994). Each monomer of the homodimeric BC contains a complete active site as opposed to residues being shared between monomers (Waldrop *et al.*, 1994). The dimeric interface is formed between the C-terminal domains of the monomers, which each contribute about 1300  $\text{\AA}^2$  of buried surface area<sup>2</sup> (Mochalkin *et al.*, 2008). The biotin carboxylase from *E. coli* is similar in structure to the apoBC of *Pseudomonas aeruginosa* (Mochalkin *et al.*, 2008) and the BC domains of *P. aeruginosa* 3-methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012), *Rhizobium etli* pyruvate carboxylase (St Maurice *et al.*, 2007), *Ruegeria pomeroyi* propionyl-CoA carboxylase (Huang *et al.*, 2010), *Saccharomyces cerevisiae*

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<sup>2</sup> Buried surface areas were calculated using the PISA program (Krissinel and Henrick, 2007) unless otherwise noted.

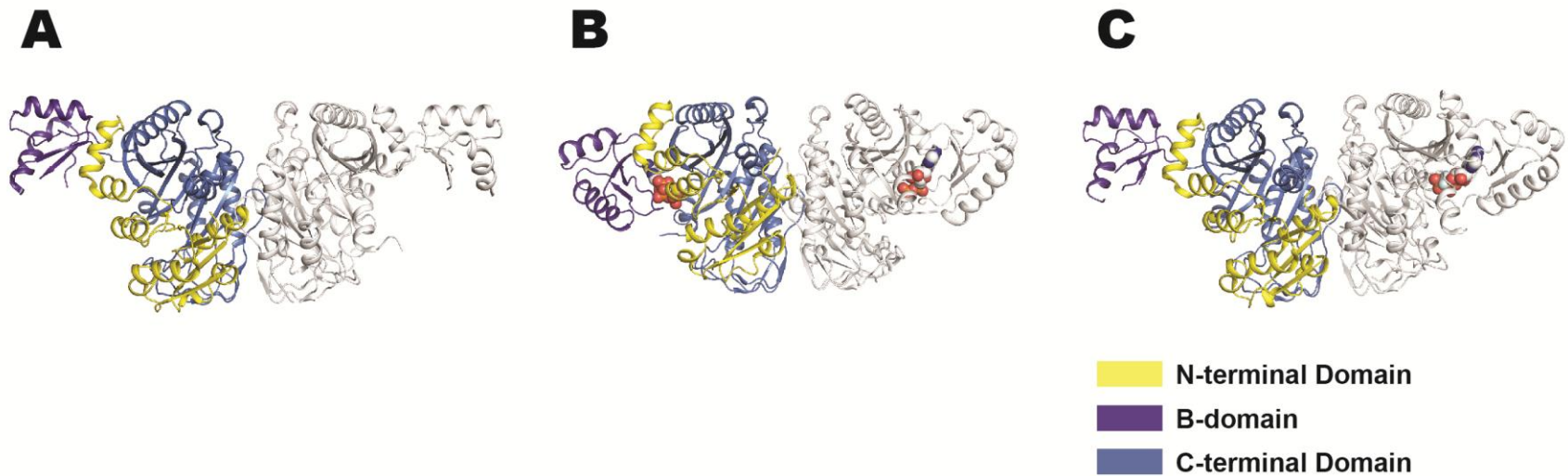


Figure 1.6 ATP-grasp domain conformations of *E. coli* biotin carboxylase. Biotin carboxylase homodimers with the left monomer color coded showing the N-terminal domain, B-domain, and C-terminal domain and the adjacent monomer in white. Atom colors are: carbon, white; nitrogen, blue; oxygen, pink; phosphate, orange and fluoride, cyan. A) open-open conformation with no bound ligands. B) closed-closed conformation with ATP bound in each active site and shown in sphere representation. C) open-closed conformation with ADPCF<sub>2</sub>P bound in the right active site and shown in sphere representation.



ACC (Shen *et al.*, 2004), and human ACC2 (Cho *et al.*, 2010) because their alpha carbon atoms superimpose with an average RMSD<sup>3</sup> of 1.35 Å and a range of 0.96-1.99 Å.

The ATP-grasp domain has been observed in both open and closed conformations. Structures of biotin carboxylase without substrate bound display an ATP-grasp domain that is splayed open from the main body of the protein (Figure 1.6 A) (Waldrop *et al.*, 2012). The BC domain from propionyl-CoA carboxylase (Huang *et al.*, 2010), 3-methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012), and pyruvate carboxylase (St Maurice *et al.*, 2007) are also observed in the open conformation in the absence of substrate. When ATP or an ATP analog binds to the monomer, the ATP-grasp domain rotates 45° to close down on top of the molecule and clasp it in the active site (Figure 1.6 B) (Waldrop *et al.*, 2012). This is referred to as the closed conformation and several structures of the closed conformation have been solved with different ligands: ATP (Thoden *et al.*, 2000), AMPPNP (Mochalkin *et al.*, 2008), AMPCP (Mochalkin *et al.*, 2008), as well as BC bound to all three substrates (Chou *et al.*, 2009), and a pyruvate carboxylase structure of the BC domain bound to BCCP (Lietzan *et al.*, 2011).

The active sites of BC have been shown to display half-sites reactivity or an extreme form of negative cooperativity where the active sites must alternate turns catalyzing the reaction (Janiyani *et al.*, 2001). This has been demonstrated by creating hybrid dimers where one monomer of BC is wild-type and the other monomer of BC was an inactive mutant. A total of four different heterodimers were made and would have been expected to show 50% of normal activity if the active sites acted independently. However, the inactive mutant monomers exerted a dominant negative effect on the wild-type monomer and the overall activity ranged from 0.4-

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<sup>3</sup> All root mean squared deviations (RMSD) were calculated using the program PDBefold (Krissinel and Henrick, 2004) unless otherwise noted.

3.6% of wild-type (Janiyani *et al.*, 2001). This is indicative that the two active sites undergo oscillating cycles of catalysis whereby when one monomer of BC is releasing product, the other monomer is binding substrates for catalysis. The structural evidence indicative of the oscillating catalytic mechanism was observed in a crystal structure where one BC monomer was bound to ADPCF<sub>2</sub>P in the closed conformation and the other monomer was in the open conformation with no molecule in the active site (Figure 1.6 C) (Mochalkin *et al.*, 2008).

Biotin carboxylase has also been shown to exhibit substrate-induced synergism (Blanchard *et al.*, 1999b). BC catalyzes the slow hydrolysis of ATP in the absence of biotin, but in the presence of biotin, the activity of BC is increased 1100-fold. ATP is thought to have several non-productive binding modes and upon the binding of biotin, ATP assumes the most favorable conformation for formation of carboxyphosphate and subsequent carboxylation of biotin (Cronan and Waldrop, 2002). Structural evidence for a non-productive binding mode was observed in the crystal structure of *E. coli* biotin carboxylase bound to AMPPNP (Figure 1.7 A) (Mochalkin *et al.*, 2008). In this structure, the  $\gamma$ -phosphate of ATP is curled back in a conformation that would not allow nucleophilic attack on the phosphate by the bicarbonate oxygen (Mochalkin *et al.*, 2008). This is in contrast to the extended conformation of the  $\gamma$ -phosphate that was observed in *Staphylococcus aureus* BC crystallized with AMPPNP and *E. coli* BC crystallized with ADPCF<sub>2</sub>P, which positions the  $\gamma$ -phosphate for the catalytic reaction (Figure 1.7 B and C) (Mochalkin *et al.*, 2008). Additionally, the extended conformation of the  $\gamma$ -phosphate is the common orientation observed in crystal structures of other enzymes from the ATP-grasp superfamily such as D-Ala:D-Ala ligase, glutathione synthetase, carbamoyl phosphate synthetase, and the BC domain of pyruvate carboxylase when bound to ATP analogs (Mochalkin *et al.*, 2008).

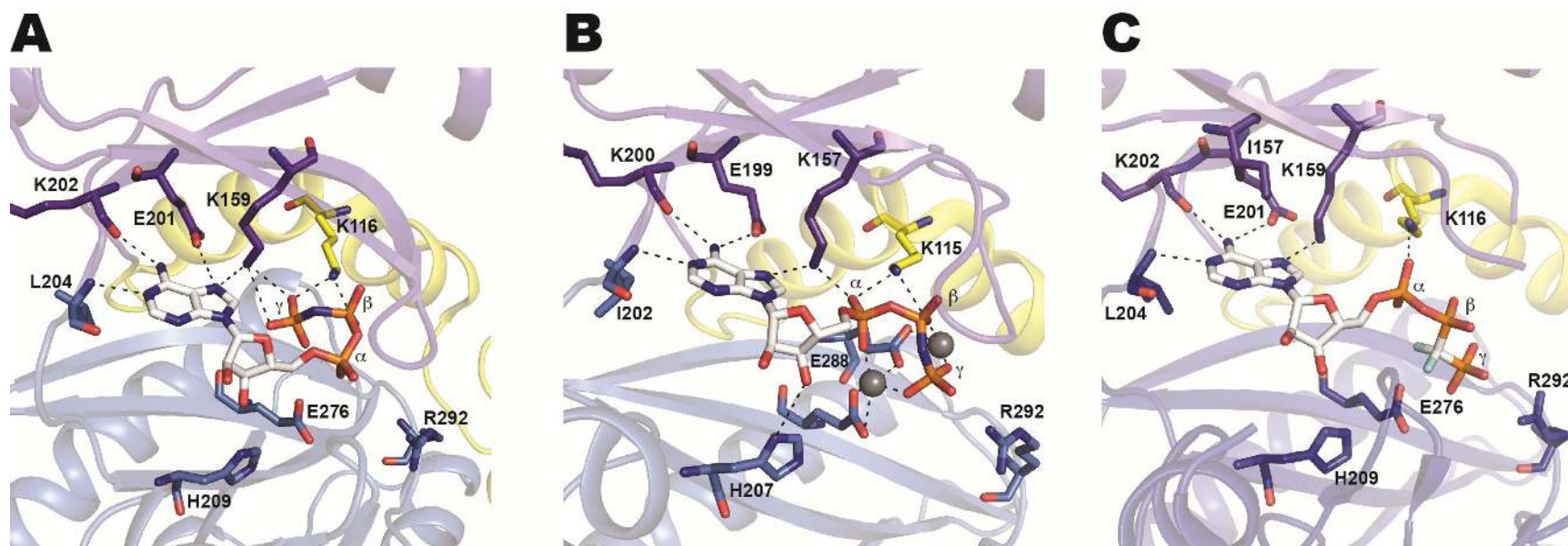


Figure 1.7 Binding of ATP analogs to biotin carboxylase. The N-terminal domain, B-domain, and C-terminal domain are shown in transparent yellow, purple, and blue, respectively. Residues are numbered to match the organism described in the figure. Black dashes indicate potential hydrogen bonds. Atom colors are: carbon, white (side chains and backbones of residues are colored to match the BC domain to which they correspond); nitrogen, blue; oxygen, red; phosphate, orange; fluoride, cyan and  $Mg^{2+}$ , grey spheres. A) *E. coli* BC bound to AMPPNP in a curled unproductive conformation. B) *S. aureus* BC bound to AMPPNP in an extended conformation bound to two  $Mg^{2+}$  ions. C) *E. coli* BC bound to ADPCF<sub>2</sub>P in an extended conformation.

Two equivalents of magnesium are required for the activity of BC (Climent and Rubio, 1986) as evidenced by the *S. aureus* structure of BC that was crystallized with AMPPNP showing two magnesium ions in the active site (Figure 1.7 B). One of the magnesium ions is coordinated to the side chains of Glu274 and Glu288 and to the  $\alpha$ - and  $\gamma$ -phosphate oxygens (Mochalkin *et al.*, 2008). The second magnesium ion was coordinated to one of the side chain oxygens of Glu288 and to oxygen atoms of the  $\beta$ - and  $\gamma$ -phosphates of AMPPNP (Mochalkin *et al.*, 2008).

### Biotin Carboxyl Carrier Protein

Early purification of BCCP yielded a range of molecular weights that were attributed to oligomerization or proteolytic cleavage of the full length protein (Fall and Vagelos, 1972). The C-terminal domain of *E. coli* BCCP has been shown to be easily isolated by proteolytic cleavage with subtilisin Carlsberg to create a stable biotinylated protein domain (BCCP<sub>sc</sub>) (Fall and Vagelos, 1973). The C-terminal domain of BCCPs are highly conserved and contain the lysine targeted for biotinylation in an AMKM motif that is invariant with few exceptions (Chapman-Smith and Cronan, 1999a). Later, Chapman-Smith *et al.* (1994) developed a system to overexpress and purify the last 87 residues of BCCP (BCCP87). BCCP87 can be biotinylated and has been shown to have an 8000-fold and 2000-fold greater catalytic efficiency than biotin in the BC and CT reactions, respectively (Blanchard *et al.*, 1999a).

The crystal structure of the C-terminal domain of biotin carboxyl carrier protein from *E. coli* was solved by Athappilly and Hendrickson (1995) (Figure 1.8) and an NMR structure was solved shortly after by Yao *et al.* (1997). The C-terminal domain comprises the last 79 residues of the 156 amino acid protein and contains a  $\beta$ -sandwich structure of two four-stranded

antiparallel  $\beta$ -sheets (Athappilly and Hendrickson, 1995). The AMKM motif containing Lys122 is biotinylated and was found in a  $\beta$ -hairpin turn that extends out from the body of the C-terminal domain (Chapman-Smith and Cronan, 1999a). In comparison, the C-terminal domains of the 1.3S subunit of transcarboxylase from *P. shermanii* (Reddy *et al.*, 1998) and the BCCP domain of human ACC2 (Lee *et al.*, 2008) superimposed over the Ca atoms of the crystal structure from *E. coli* with RMSDs of 1.53 and 1.60 Å, respectively. The major difference between the structures was the lack of a “thumb” or short loop of about eight amino acids that projects away from the body of the C-terminal domain in *E. coli* BCCP (Figure 1.9) (Cronan, 2001).

The role of the “thumb” of *E. coli* BCCP is still unknown but it is thought to interact with the biotin moiety and to be involved in protein-protein interactions (Cronan, 2001). The thumb is unlikely to be involved in the biotinylation reaction because the BPL from *E. coli* will biotinylate proteins that lack a thumb (Chapman-Smith and Cronan, 1999b). The thumb region of BCCP was shown to be essential for growth and function because expressing BCCP mutants with a deleted thumb region or replacing the thumb with an alanine, prevented culture growth (Cronan, 2001). A crystal structure of biotinylated BCCP showed the biotin moiety curled back and hydrogen bonding with the side chain and carboxylate of Thr94 located in the thumb (Athappilly and Hendrickson, 1995). The NMR structure of BCCP87 also showed the biotin prosthetic group curled back and interacting with residues 95-100 in the thumb region of the protein (Roberts *et al.*, 1999). Therefore, the thumb may be involved in immobilization of the biotin in solution.

The orientation of the residues in the C-terminal domain of BCCP affects efficient use of the protein as a substrate. The C-terminal domain is thought to undergo a slight change upon biotinylation because the unbiotinylated protein interacts with fluorescein-5-maleimide to form a

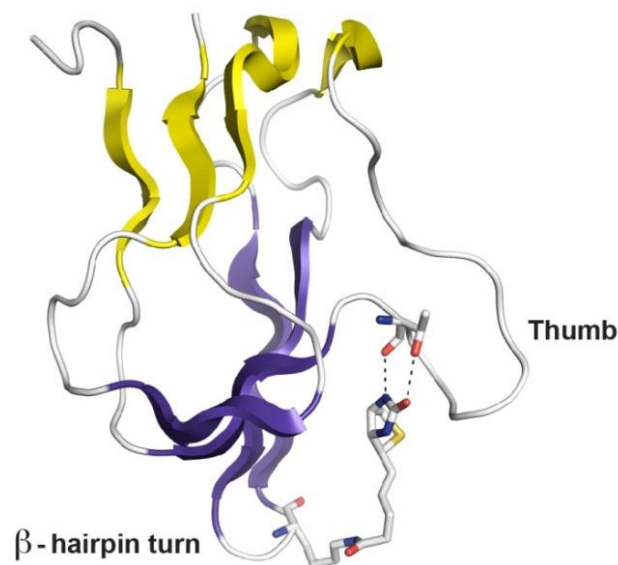


Figure 1.8 C-terminal domain of the biotin carboxyl carrier protein.  $\beta$ -sandwich structure is shown as two sets of four strands in yellow and purple. The biotin moiety attached to lysine 122 is curled back and interacting with the carbonyl oxygen and side chain hydroxyl of threonine 94. Atom colors are: carbon, white; nitrogen, blue; oxygen, red and sulfur, yellow.

thioether bond at the single cysteine, C116, of the BCCP87 protein while the biotinylated BCCP resists this modification (Chapman-Smith *et al.*, 1997). The NMR structure of apo- (unbiotinylated) and holo- (biotinylated) BCCP87 showed that the side chain packing in holoBCCP87 was more favorable than that observed in apoBCCP87 and could be interpreted as why the biotinylated C-terminal domain is more stable and resistant to proteolysis (Roberts *et al.*, 1999). Additionally, mutations of the methionines on either side of Lys122 in *E. coli* to lysine still allowed ~50% biotinylation of the protein *in vivo* and changing the MKM to KAM or MAK abolished biotinylation (Reche *et al.*, 1998). However, changing the methionines flanking the lysine in the 1.3S subunit of transcarboxylase still allowed biotinylation but carboxylation of

biotin was 0-40% of normal activity (Shenoy *et al.*, 1988). Additionally, ~35 residues on both sides of the biotin attachment site are required for biotinylation (Chapman-Smith and Cronan, 1999a).

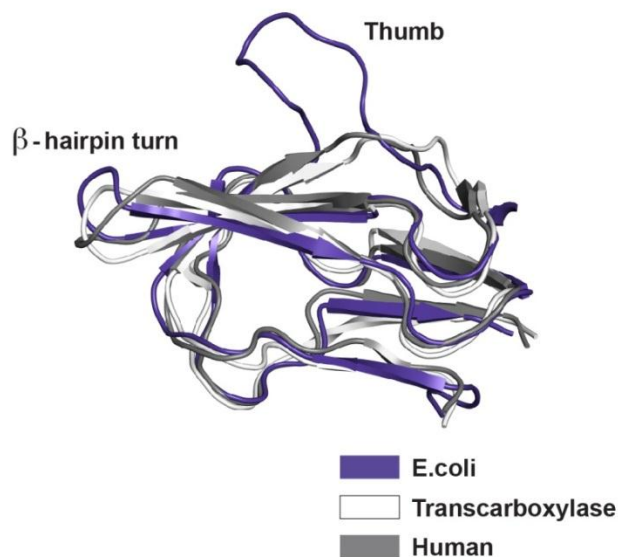


Figure 1.9 Superposition of biotin carboxyl carrier protein from *E. coli* (PDB ID= 1BDO), *P. shermanii* transcarboxylase and BCCP domain of human ACC2.

The N-terminal domain of *E. coli* BCCP has not been crystallized and was not observed in the NMR structures, which is thought to be the result of a pro/ala rich linker region between the domains that causes the N-terminal domain to be highly flexible (Cronan, 2002). This domain is thought to be involved in interactions between subunits and possibly formation of a holo-complex (Li and Cronan, 1992a). Nenortas and Beckett (1996) and Choi-Rhee and Cronan (2003) showed that when BCCP was expressed in the presence of BC, these proteins formed a stable complex. Furthermore, purification of BCCP was made easier by expression in the presence of BC and then separating the BCCP from BC using 2 M urea (Nenortas and Beckett,

1996). BCCP alone has a tendency to form large aggregates, which has made it difficult to confidently determine the oligomeric state of the protein in solution (Choi-Rhee and Cronan, 2003; Nenortas and Beckett, 1996). The N-terminal domain appears to be responsible for aggregation because sedimentation equilibrium experiments indicated that BCCP87 was a monomer in solution while BCCP formed 4-10 mers (Nenortas and Beckett, 1996). Additionally, the first 18 residues of the N-terminal domain of the 1.3S subunit of transcarboxylase from *P. shermanii* were shown to be required for formation of a stable complex with the 5S and 12S subunits (Shenoy *et al.*, 1993).

Lastly, rotation about the C $\alpha$  atom of the biotinylated lysine would allow a maximum ~30 Å of movement (Figure 1.10) (Huang *et al.*, 2010; St Maurice *et al.*, 2007). However, crystal structures of pyruvate carboxylase (St Maurice *et al.*, 2007), propionyl-CoA carboxylase (Huang *et al.*, 2010) and 3-methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012) have BC to CT active site distances of 75, 55, and 80 Å, respectively. This means that BCCP must translocate to cover the extra distance between active sites and evidence of this BCCP domain movement was observed in the cryo-electron microscopy structures of human propionyl-CoA carboxylase (Huang *et al.*, 2010).

### Carboxyltransferase

The carboxyltransferase subunit was the last of the three components of ACC to be crystallized. The CT domain from yeast was crystallized first (Zhang *et al.*, 2003) and the crystal structures of *S. aureus* and *E. coli* CT followed shortly thereafter (Figure 1.11) (Bilder *et al.*, 2006). The bacterial CTs form an  $\alpha_2\beta_2$  heterotetramer and the *E. coli* and *S. aureus* CT subunits share 52% sequence identity (Bilder *et al.*, 2006). The *E. coli*  $\alpha$ -subunit is composed of 319



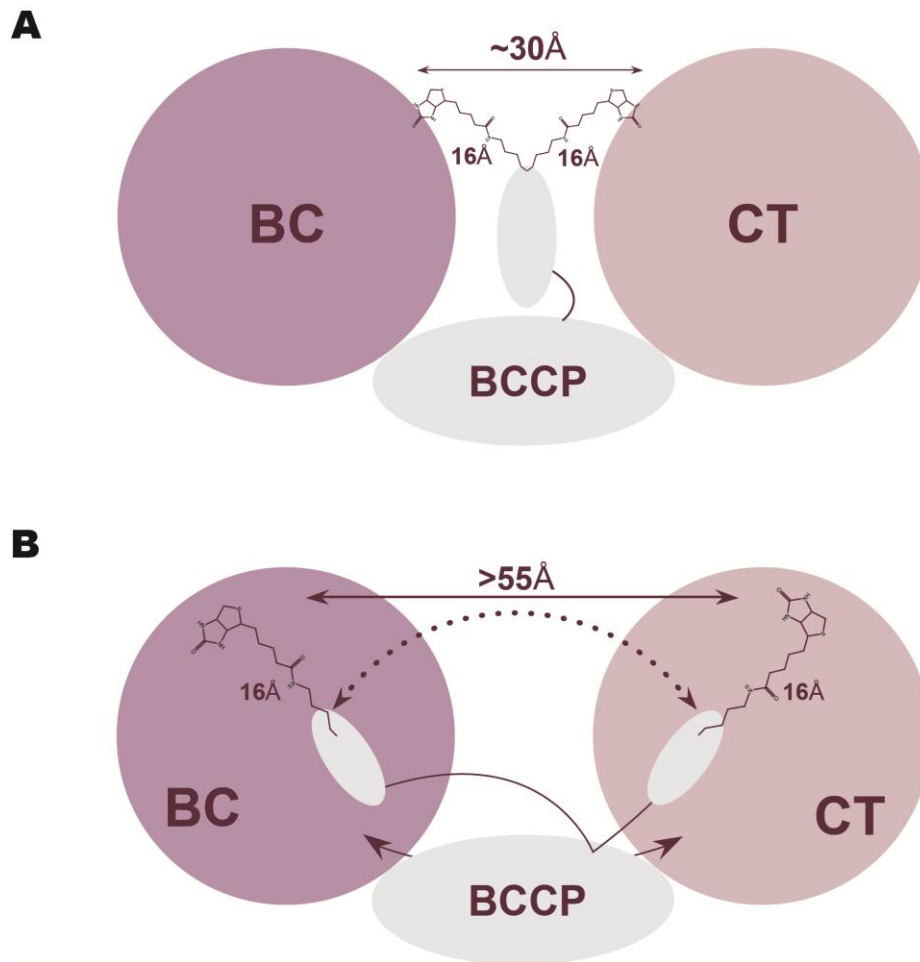


Figure 1.10 Models of biotinylated lysine and BCCP movement in biotin-dependent carboxylases. A) “swinging arm” model where the 16 Å biotinylated lysine rotates freely on a fixed biotin carboxyl carrier protein. This model would allow about 30 Å of total movement. B) “translocation” model where the C-terminal domain of BCCP moves freely between active sites via the pro/ala rich linker region while the N-terminal domain interacts with BC and CT.

amino acids and the  $\beta$ -subunit contains 304 amino acids (Bilder *et al.*, 2006). Both the  $\alpha$ - and  $\beta$ -subunits are made up of a core of repeating  $\beta$ - $\beta$ - $\alpha$  motifs and the N-terminus of the  $\beta$ -subunit contains a  $C_4$  zinc finger (Waldrop *et al.*, 2012). The two active sites are formed where the  $\alpha$ - and  $\beta$ -subunits come together at two  $9000 \text{ \AA}^2$  interfaces on opposite sides of the promoter (Bilder *et al.*, 2006). The  $\alpha$ -subunit of carboxyltransferase binds biotin and the  $\beta$ -subunit binds acetyl-CoA (Bilder *et al.*, 2006).

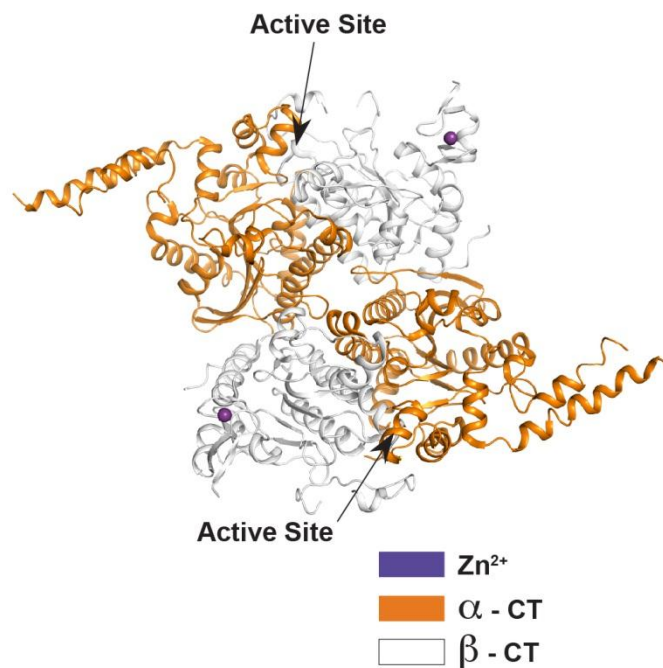


Figure 1.11 Cartoon representation of the carboxyltransferase subunit of *E. coli*.

The symmetrical core folds of the  $\alpha$ - and  $\beta$ -subunits were shown to belong to the crotonase superfamily of enzymes (Figure 1.12) (Bilder *et al.*, 2006). Crotonase folds are used to stabilize an enolate anion via an oxyanion hole (Hamed *et al.*, 2008). The oxyanion hole is formed from two peptidic NH groups located at the N-terminus of an  $\alpha$ -helix to putatively use the helix dipole for better stabilization of the anion (Figure 1.12) (Waldrop *et al.*, 2012). The conserved active site residues that putatively form the oxyanion holes in *E. coli* CT are Gly206 and 207 in the  $\alpha$ -subunit and Gly204 and 205 in the  $\beta$ -subunit (Figure 1.12) (Bilder *et al.*, 2006). The similar folds in the  $\alpha$ - and  $\beta$ -subunits of CT likely arose from an early evolutionary gene duplication event that gave rise to similar functions for the two proteins (Waldrop, 2011). The purpose of the similar folds of the domains becomes clear after consideration of the catalytic mechanism of carboxyltransferase. The reaction necessitates that a proton be removed from

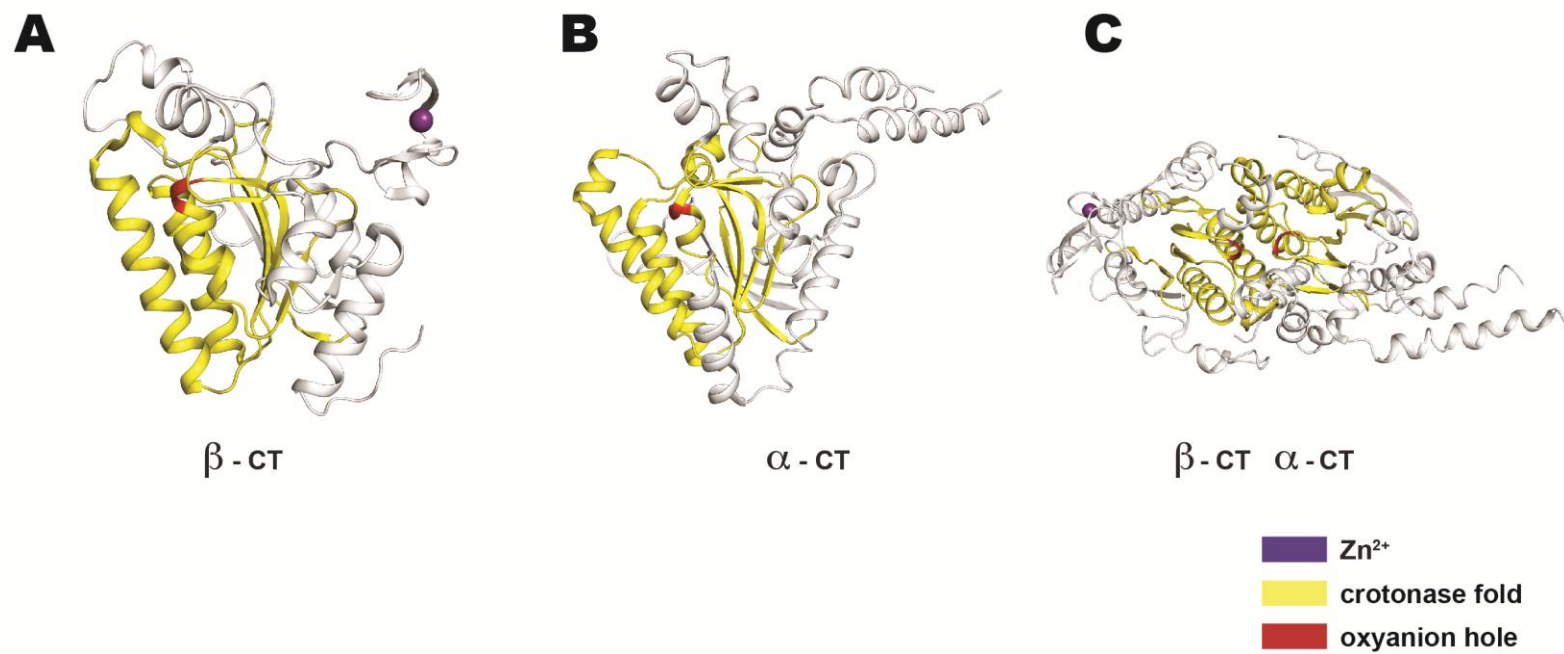


Figure 1.12 The crotonase folds and active site of the  $\alpha$ - and  $\beta$ -subunits of *E. coli* carboxyltransferase.

acetyl-CoA to form an enolate anion. The base for removal of the proton is currently unknown. It could be that an active site glutamate is what removes the proton as observed in the other crotonase family enzymes (Hamed *et al.*, 2008), or once biotin is decarboxylated, the imidate anion of biotin could abstract the proton (Waldrop *et al.*, 2012). Both scenarios allow for the nucleophilic attack of the enolate on the carbon atom of CO<sub>2</sub>. The crotonase fold of the  $\alpha$ - and  $\beta$ -subunits is required to stabilize the imidate and enolate forms of biotin and the acetyl-CoA substrates, respectively (Figure 1.13) (Waldrop, 2011).

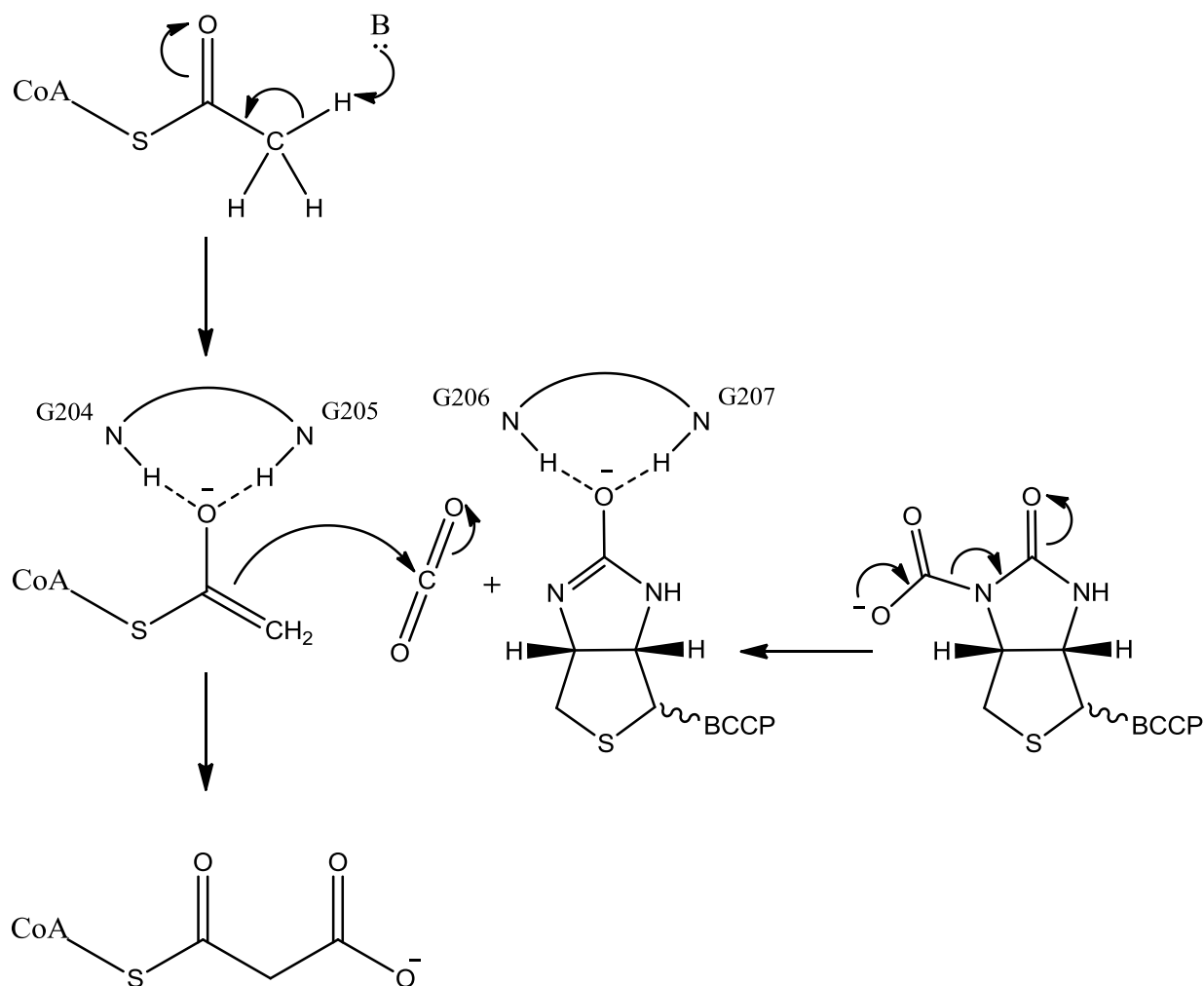


Figure 1.13 A proposed mechanism of the *E. coli* carboxyltransferase reaction.

The two subunits of bacterial CT are fused together as two domains on the same polypeptide in yeast, the 12S subunit of transcarboxylase from *P. shermanii*, the  $\beta$ -subunit of *Roseobacter denitrificans* propionyl-CoA carboxylase (Huang *et al.*, 2010), and the  $\beta$ -subunit of *P. aeruginosa* 3-methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012), and each domain of these enzymes also contains the crotonase fold (Waldrop *et al.*, 2012). In these multidomain proteins, the N-terminal domain binds the acyl-CoA substrate and the C-terminal domain binds to biotin (Waldrop *et al.*, 2012). The active sites are formed in a head to tail fashion with the N-terminal domain interacting with the C-terminal domain of a neighboring subunit (Waldrop *et al.*, 2012). Yeast CT comes together to form a dimer (Zhang *et al.*, 2003). In contrast, the  $\beta$ -subunits of propionyl-CoA carboxylase (Huang *et al.*, 2010) and 3-methylcrotonyl-CoA (Huang *et al.*, 2012) carboxylase and the 12S subunit of transcarboxylase (Waldrop *et al.*, 2012) form N- to C-terminal dimers of dimers, which is common in the crotonase superfamily (Hamed *et al.*, 2008).

The major difference between the bacterial and plant chloroplast multisubunit carboxyltransferases and homologous carboxyltransferases from other organisms is the presence of the C<sub>4</sub> zinc finger observed in the former (Figure 1.14) (Bilder *et al.*, 2006). A common function of zinc fingers is to bind nucleic acids. The CT from *E. coli* was shown to bind DNA non-specifically as well as the DNA analog heparin (Benson *et al.*, 2008). Shortly after, CT was shown to specifically bind mRNA coding for the  $\alpha$ - and  $\beta$ -subunits of CT (Meades *et al.*, 2010). Furthermore, the binding of acetyl-CoA and mRNA was shown to be mutually exclusive and mutation of the zinc domain cysteines abolished nucleic acid binding and catalytic activity (Meades *et al.*, 2010).

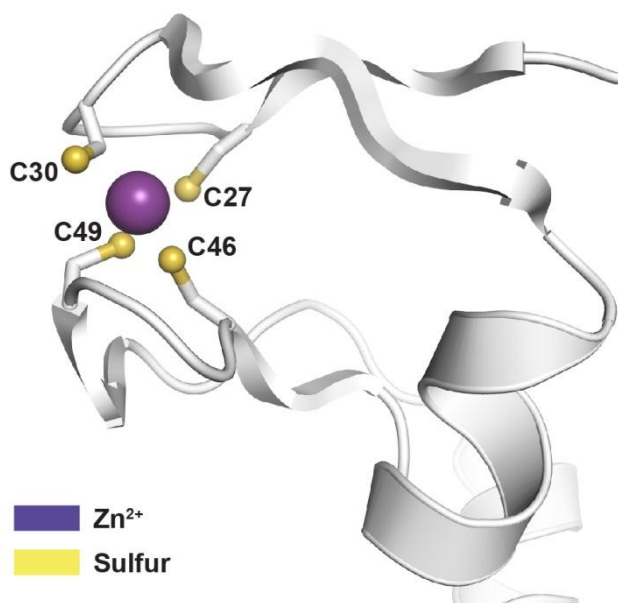


Figure 1.14 The zinc domain of the carboxyltransferase subunit from *E. coli*.

## Regulation of Acetyl-CoA Carboxylase

### Prokaryotic

Acetyl-CoA carboxylase has been shown to be the rate-limiting and committed step in fatty acid biosynthesis and is therefore ideal for regulation of this pathway (Davis *et al.*, 2000). Fatty acid synthesis accounts for 94% of the ATP consumed in membrane biogenesis and therefore inefficient regulation of this pathway would result in a detrimental waste of cellular energy (Li and Cronan, 1993). Furthermore using northern blot analysis, it has been shown that there is a direct correlation between culture growth rate and mRNA expression of the ACC genes in *E. coli* (Li and Cronan, 1993) and the ratio of the levels of growth rate to transcription of the *accBC* gene in the Gram-positive organism *Bacillus subtilis* are similar (Marini *et al.*, 2001).

The four ACC genes may be transcriptionally regulated. Overexpression of BCCP was shown to reduce *accBC* transcript levels by 90% 40 min after induction compared to uninduced levels (James and Cronan, 2004). The overexpression of the first 44 residues of the N-terminal domain of BCCP decreases mRNA transcripts by 54% in 30 min and 96% by 60 min (James and Cronan, 2004). Additionally, James and Cronan (2004) replaced the promoter of the *accBC* mini-operon with the promoter from the lactose operon. Transcription of the *accBC* gene with the lactose promoter increased 4.5-5 fold in comparison to transcription in the presence of the wild-type promoter (James and Cronan, 2004). The authors concluded that BCCP autoregulates the transcription of the *accBC* operon (James and Cronan, 2004). This is likely the method of regulation in many bacterial species because the cotranscription of these two genes is highly conserved (James and Cronan, 2004). In contrast, the *accA* and *accD* genes encoding the  $\alpha$ - and  $\beta$ -subunits of CT, respectively, are expressed in gene clusters on opposite sides of the *E. coli* chromosome (Li and Cronan, 1993). For instance, the gene for the  $\alpha$ -subunit of carboxyltransferase begins within the coding sequence of the *polC* gene encoding the catalytic subunit of DNA polymerase III (Li and Cronan, 1992b). Both genes are transcribed in a clockwise rotation and could be coregulated; however, there is no evidence to support this hypothesis (Li and Cronan, 1992a, 1993).

Recently, CT has been shown to regulate its own translation by specifically binding the mRNA coding for  $\alpha$ - and  $\beta$ -CT subunits (Meades *et al.*, 2010). Acetyl-CoA competes with mRNA for the active site of CT (Meades *et al.*, 2010). This allows CT to work as a “dimmer switch” for its own translation by sensing the cellular levels of acetyl-CoA (Meades *et al.*, 2010). Therefore, when cellular growth rate increases and the intracellular levels of acetyl-CoA are

high, CT is displaced from its mRNA allowing the production of malonyl-CoA and translation of CT (Figure 1.15) (Meades *et al.*, 2010).

The end product of fatty acid synthesis, acylated acyl carrier protein, is a negative feedback inhibitor of ACC (Davis and Cronan, 2001). These authors showed that 40  $\mu\text{M}$  of acyl-ACP (the acyl group being 6 to 20 carbon units) inhibits ACC activity up to 70% (Davis and Cronan, 2001).

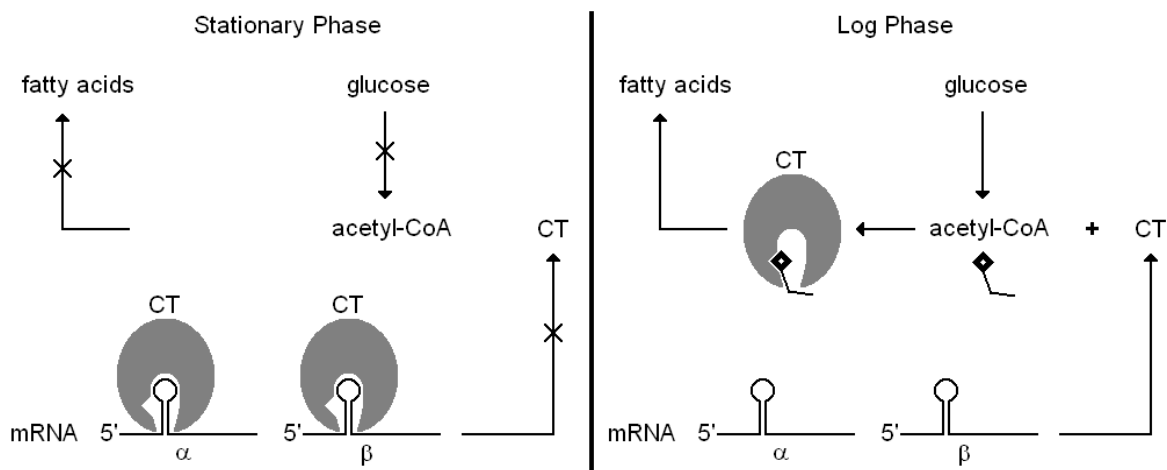


Figure 1.15 Regulation of bacterial carboxyltransferase activity and translation. (Figure taken from Meades *et al.* (2010))

## Eukaryotic

Both isoforms of eukaryotic ACC have been shown to be similarly controlled at the transcriptional level. For example, human ACC1 has three promoters (PI, PII and PIII). The PI promoter is constitutively active to provide basal levels of fatty acid synthesis (Mao *et al.*, 2003). Several transcription factors are involved in activating transcription of the gene from the PII promoter, the most notably being sterol regulatory-element binding protein (SREBP), carbohydrate response element binding protein, and triiodothyronine (T3 or a thyroid hormone)



(Mao *et al.*, 2003; Tong, 2005). In cell lines transfected with each ACC1 promoter upstream of a luciferase reporter gene, only PII was shown to be activated by SREBP-1a (one of three isoforms of SREBP), which was indicated by a 2.5- to 4-fold increase in luciferase activity (Mao *et al.*, 2003). T3 had a similar effect on only the PII promoter and luciferase activity was increased 78-fold (Mao *et al.*, 2003). The PIII promoter is expressed in lactating and cancerous mammary glands (Mao *et al.*, 2003). This tissue specific transcription allows for expression of ACC in cells that require the production of lipids for neonatal development (Mao *et al.*, 2003).

Before covering activation and post-translational modifications of ACC1 and ACC2, it is important to understand the physiological role of ACC2. The main difference between ACC1 and ACC2 is the longer N-terminal region of ACC2, which allows this protein to anchor to the outer membrane of the mitochondria (Abu-Elheiga *et al.*, 2000). ACC2 colocalizes to the outer membrane of the mitochondria on the cytoplasmic side in proximity to the carnitine palmitoyltransferase I (CPTI), which transports acyl-CoAs into the mitochondria for  $\beta$ -oxidation (Abu-Elheiga *et al.*, 2000; Kim, 1997). CPTI is inhibited by malonyl-CoA in the nanomolar range and when ACC2 is inactivated,  $\beta$ -oxidation is initiated to restore cellular energy levels (Kim, 1997; Tong, 2005; Wakil and Abu-Elheiga, 2009).

The ACC1 and ACC2 isoforms are allosterically regulated by citrate, isocitrate, malonate, sulfate and phosphate (Tong, 2005; Zu *et al.*, 2013). Citrate is an allosteric activator of ACC ( $K_a \sim 2\text{mM}$ ) and causes polymerization of the mammalian enzyme resulting in 10-20 ACC dimers associating to form an 8000 kDa complex (Tong, 2005). In *S. cerevisiae* citrate activates the homotetrameric enzyme but does not cause polymerization and in contrast, citrate does not activate ACC in the yeast *Schizosaccharomyces pombe* (Tong, 2005). Lastly, similar to the

bacterial ACCs, mammalian ACC1 and ACC2 exhibited negative feedback inhibition by long-chain acyl-CoAs from the end of fatty acid synthesis (Munday, 2002).

Both ACC1 and ACC2 can be post-translationally modified by phosphorylation. ACC1 and ACC2 are deactivated when phosphorylated by AMP-activated kinase (AMPK) (Kim, 1997). AMPK phosphorylates serines 79, 1200, and 1215 in ACC1 and 218 in ACC2 resulting in an 80% decrease in  $V_{\max}$  (Munday, 2002). Phosphorylation of Ser79 and Ser218 may be the sites of the modifications that are necessary to deactivate the enzymes due to the fact that ACC2 does not have homologous serine 1200 or 1215 phosphorylation sites (Tong, 2005). AMPK is activated by a variety of stress signals and exercise which ultimately shuts down fatty acid synthesis and initiates  $\beta$ -oxidation of fatty acids (Tong, 2005). Protein kinase A (PKA) phosphorylates serine 77 in ACC1 and ACC2 as well as 1200 in ACC1 but the purpose of this covalent modification is not well understood (Zu *et al.*, 2013). The literature on this subject matter varies, but phosphorylation of ACC at these sites appears to result in a ~15% decrease in the  $V_{\max}$  of the enzyme (Munday, 2002). The decreases in  $V_{\max}$  probably result from phosphorylation of serine 77 or 79. Studies using PKA or AMPK to phosphorylate their respective serines showed the expected decrease in activity of ACC (Davies *et al.*, 1990). Removal of the N-terminal serine 77 and 79 from rat liver ACC using trypsin after incubation with PKA or AMPK increased the  $V_{\max}$  of phosphorylated ACC 1.5-fold and 3.5-fold, respectively (Davies *et al.*, 1990). Furthermore, the PIII promoter transcribes an ACC1 that is lacking the N-terminal phosphorylation sites and this enzyme is thought to be constitutively active (Tong, 2005).

The mechanisms for activation and deactivation of ACC work against each other to control fatty acid synthesis and  $\beta$ -oxidation in the cell. Citrate has a reduced affinity for ACC

when it is phosphorylated and protects ACC from phosphorylation through polymerization (Munday, 2002). Ultimately, regulation at the protein level allows activation of ACC when nutrients are high leading to higher concentrations of acetyl-CoA, citrate and ATP; however, activities leading to depletion of cellular energy and increased AMP, like exercise, activate AMPK and deactivate ACC, initiating  $\beta$ -oxidation of fatty acids (Wakil and Abu-Elheiga, 2009).

## **Antibiotic Resistance and Acetyl-CoA Carboxylase as a Target for Antibiotics**

### Background

Antibiotic resistance is a growing problem worldwide. Pathogenic and non-pathogenic bacteria are both problematic because some resistance mechanisms can be transferred between microorganisms (Gilchrist *et al.*, 2007; Gould, 2009). Overprescription and non-therapeutic use of antibiotics (i.e. routine addition of antibiotics to feeding and procedures in the livestock industry) are two of the main causes of rising resistance and reversing the practices we are entrenched in remains a problem (Gilchrist *et al.*, 2007; Gould, 2009). Furthermore, the production of new and novel antibiotics is not keeping up with resistance and threatens to render our current arsenal of antibiotics ineffective (Costelloe *et al.*, 2010; Gould, 2009; Katz *et al.*, 2006).

### Fatty Acid Synthesis as an Antibiotic Target

Triclosan or 2,4,4'-trichloro-2'-hydroxy-diphenyl ether epitomizes the misuse of antibiotics as well as validates the enzymes of fatty acid synthesis as a viable antibiotic target (Figure 1.16, 1) (Schweizer, 2001). Triclosan is the active ingredient in antibacterial products including soap, toothpaste, cosmetics and is infused in children's toys, bedding, clothes and other

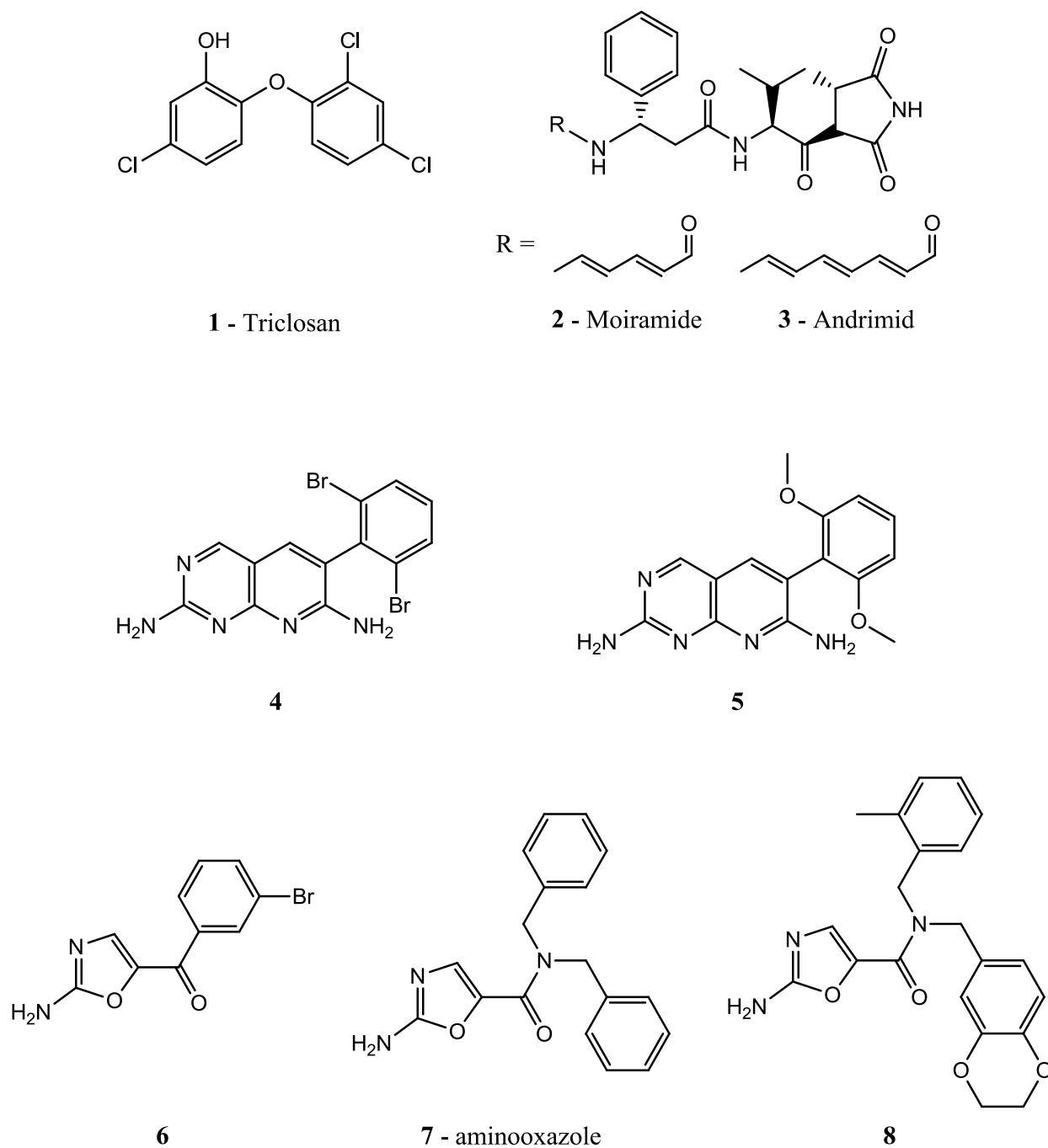


Figure 1.16 Inhibitors of enzymes involved in type II fatty acid synthesis. **1** enoyl-ACP reductase inhibitor. **2** and **3** are pyrrolidinedione inhibitors of carboxyltransferase. **4** and **5** are pyridopyrimidine inhibitors of biotin carboxylase. **6-8** are aminooxazole inhibitors of biotin carboxylase.

common household items. Triclosan inhibits enoyl-ACP reductase (EAR), an enzyme involved in fatty acid synthesis downstream of ACC, within the picomolar range (Schweizer, 2001). However, the excessive overuse of this antibiotic has contributed to several mechanisms of resistance, which include mutations in EAR, increased expression of EAR, efflux pumps to expel the molecule from the organism and degradation of the molecule (Schweizer, 2001). Therefore, new antibiotic targets are required.

#### Targeting the Carboxyltransferase Active Site

Acetyl-CoA carboxylase was validated as an antibiotic target when Freiberg *et al.* (2004) demonstrated that pseudopeptide pyrrolidinediones, such as moiramide B and andrimid, inhibit the carboxyltransferase subunit of bacterial ACC with nanomolar affinity (Figure 1.16, **2** and **3**). These compounds were shown to effectively inhibit the CT subunit of Gram-negative and Gram-positive microorganisms including *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *Streptococcus pneumoniae* while not inhibiting the human isoform of the enzyme (Freiberg *et al.*, 2004; Freiberg *et al.*, 2006). Kinetic analysis showed that the natural products moiramide B and andrimid are competitive inhibitors versus malonyl-CoA (Freiberg *et al.*, 2004). However, there are currently no structures of bacterial CT in complex with inhibitors or substrates and these structures are desperately needed to continue development of CT as an antibiotic target.

#### Targeting the Biotin Carboxylase Active Site

Scientists at Pfizer described the first class of compounds with antibiotic activity targeting the biotin carboxylase subunit by using whole bacterial-cell screening with a compound library originally made to target eukaryotic protein kinases (Miller *et al.*, 2009). Pyridopyrimidine compounds, such as **4** and **5** in Figure 1.16, exhibited potent antibacterial

activity against several human pathogens (Miller *et al.*, 2009). Mutations responsible for resistance to high concentrations of the pyridopyrimidine compounds were mapped to the gene

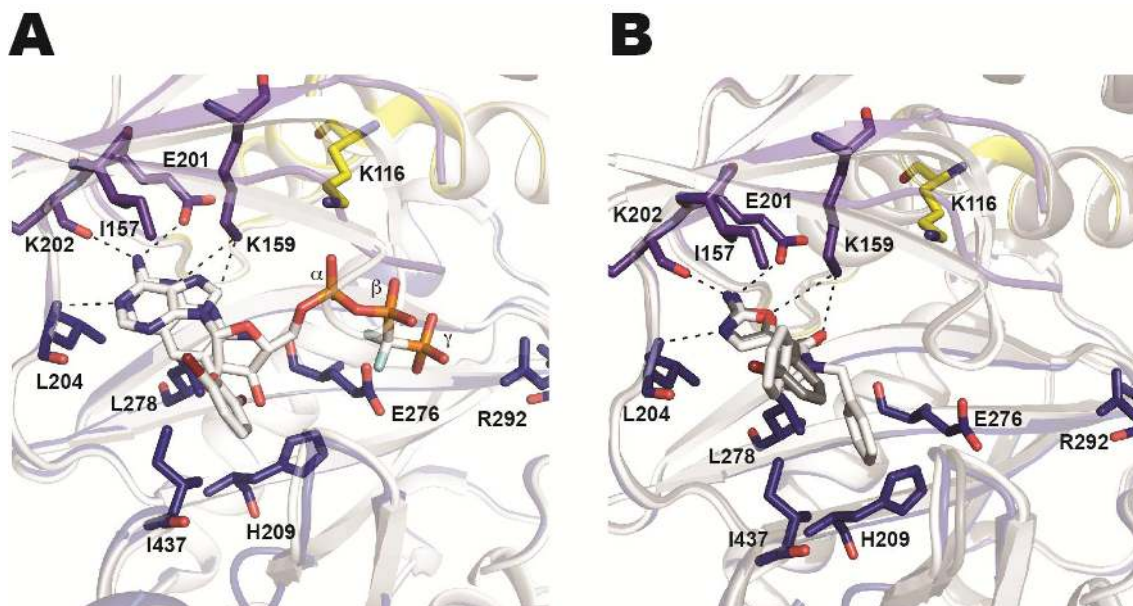


Figure 1.17 Overlays of inhibitors bound to *E. coli* biotin carboxylase. Structures of *E. coli* BC bound to inhibitors with antibiotic properties are shown superimposed over *E. coli* BC bound to AMPPCF<sub>2</sub>P. The structure bound to the ATP analog is shown with the N-terminal domain, B-domain, and C-terminal domain as well as their corresponding residues represented as sticks in yellow, purple, and blue, respectively. The residues are represented in their single letter abbreviation and their sequence number in *E. coli* BC. Atom colors are: carbon, white or dark grey (side chains and backbones of residues are colored to match the BC domain to which they correspond); nitrogen, blue; oxygen, red; phosphate, orange; fluoride, cyan and bromine, dark red. Hydrogen bonding interactions are shown as dashed lines. A) Shows compound 4 bound to BC (in white transparency) overlaid onto BC bound to AMPPCF<sub>2</sub>P. B) Shows the structures of BC bound to compound 6 and compound 7 overlaid on BC bound to AMPPCF<sub>2</sub>P. The ATP analog has been removed for clarity and compound 6 is shown with dark grey carbons to differentiate it from compound 7 shown with white carbons. The cartoon ribbons of BC match their ligand carbon color.

encoding the BC subunit of ACC. The pyridopyrimidine hits were found to inhibit BC with an IC<sub>50</sub> in the nanomolar range (Miller *et al.*, 2009). Furthermore, the compounds show selectivity for BC over 30 other eukaryotic protein kinases and rat liver ACC. Finally, several crystal

structures of BC in complex with pyridopyrimidines have been solved. Cocrystallization of compound **4** with BC showed that it bound to the ATP site (Figure 1.17 A) (Miller *et al.*, 2009).

Compound **4** was used as a template in ligand-based virtual screening to identify new inhibitors of BC. These compounds, such as **6**, had similar pharmacophore features and antimicrobial properties to the pyridopyrimidines (i.e. special arrangement of functional groups) (Mochalkin *et al.*, 2009). Compound **6** contained an exo-cyclic amino group in the oxazole ring and had an  $IC_{50}$  for BC in the micromolar range (Figure 1.16, **6**) (Mochalkin *et al.*, 2009). A subsequent crystal structure of BC bound to compound **6** showed that it bound the ATP site similar to compound **4**. Using fragment-based drug design, Mochalkin *et al.* (2009) optimized binding interactions of the inhibitor with BC and extended the original scaffold of compound **6** into a hydrophobic pocket near the ribose binding region of the ATP site. This approach yielded derivatives from compound **6** such as compound **7** and compound **8** that had 200- and 3000-fold more potent  $IC_{50}$  values for BC than compound **6** (Figure 1.16, **7** and **8**) (Figure 1.17 B) (Mochalkin *et al.*, 2009).

### The Future of New Antibiotic Targets

Despite efforts over the last couple of years, there are currently no antibiotics in clinical use that target ACC. Even worse, pharmaceutical company's interest in pursuing profitable drug development has created an insufficient level of antibiotic research (Table 1.1) (Katz *et al.*, 2006). However, this provides an opportunity for academic researchers to pick up where the pharmaceutical companies left off. Determining new antibiotic targets and bringing existing inhibitors closer to being clinically useful could create incentive for these companies to rejoin the fight against drug resistant pathogens. Development of antibiotics could stop microorganisms

like *M. tuberculosis* which cause an estimated 8 million new infections and 2 million deaths worldwide each year (Lin *et al.*, 2006).

Table 1.1 Antibiotic development at select pharmaceutical companies in 2006*			
Company	New antibiotic approved for use since 1998 in 2006	Number of antibiotics in phase 2 trials or beyond in 2006	Approximation of total drugs in phase 2 trials or beyond in 2006
Pfizer	2	2	16
Merck & Co.	1	1	26
Johnson & Johnson	0	1	18
GlaxoSmithKline	0	1	34
Bristol-Myers Squibb	0	0	8
Novartis	0	0	41
Bayer	0	0	4

\*The data in Table 1.1 was reconstructed from Katz *et al.* (2006).

## Purpose of Dissertation

Despite over 50 years of research on *E. coli* acetyl-CoA carboxylase, it has never been established if the three components, biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase work in isolation or come together to form a macromolecular complex. Furthermore, there has never been a three-dimensional structure of acetyl-CoA carboxylase or biotin carboxyl carrier protein bound to BC or CT. The focus of this dissertation is to determine if the individual components of acetyl-CoA carboxylase form a complex, and if the complex formation plays a role in regulation of enzyme activity. Chapter 2 describes complex formation of *E. coli* acetyl-CoA carboxylase using enzyme kinetic, co-immunoprecipitation or pull-down assays, and *in vivo* cooverexpression of all three components. Chapter 3 describes the first complex of biotin carboxylase and biotin carboxyl carrier protein subunits of *E. coli* acetyl-CoA carboxylase to a 2.49 Å resolution.



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## CHAPTER 2

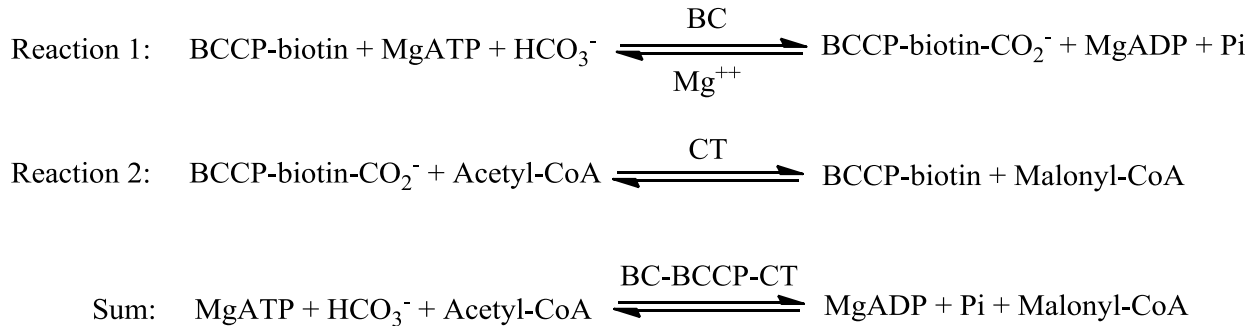
### COMPLEX FORMATION AND REGULATION OF *ESCHERICHIA COLI* ACETYL-COA CARBOXYLASE

#### Introduction

Fatty acid biosynthesis is one of the most important and fundamental metabolic pathways in nature. Animals use fatty acids as a component of cellular membranes and for energy storage, while bacteria only use fatty acids for membrane biogenesis (Zhang and Rock, 2008). Thus, regulation of fatty acid biosynthesis is critical to the survival of organisms from bacteria to humans. Fatty acid synthesis in bacteria is commonly referred to as fatty acid synthesis II (FAS II) to distinguish it from the eukaryotic pathway, which is designated fatty acid synthesis I (FAS I). FAS II is distinguished from FAS I by the fact that all of the enzymes in the pathway are separate proteins whereas in FAS I, the enzymes constitute domains on a single polypeptide (Rock and Cronan, 1996). The major regulatory enzyme in bacterial fatty acid biosynthesis is acetyl-CoA carboxylase (ACC), which catalyzes the committed step in this pathway (Cronan and Waldrop, 2002).

Acetyl-CoA carboxylase catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA via two half-reactions (Scheme 2.1). In *Escherichia coli* and many other Gram-negative and Gram-positive bacteria, the enzyme consists of three different components: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). In the first half-reaction, biotin carboxylase catalyzes the formation of a reactive carboxyphosphate intermediate through the ATP-dependent phosphorylation of bicarbonate (Knowles, 1989). The carboxyl group is transferred from carboxyphosphate to the vitamin biotin, which *in vivo* is covalently attached to BCCP (Athappilly and Hendrickson, 1995; Chapman-Smith and Cronan, 1999). In the second half-reaction, carboxyltransferase transfers the carboxyl

group from carboxybiotin to acetyl-CoA to make malonyl-CoA (Blanchard and Waldrop, 1998; Polakis *et al.*, 1974).



Scheme 2.1 Acetyl-CoA carboxylase half-reactions and sum.

While *E. coli* acetyl-CoA carboxylase consists of three different proteins, four different gene products are required to make the functional enzyme. The biotin carboxylase component is a homodimer with a monomer molecular weight of 49.4 kDa and is encoded by the *accC* gene. The BCCP component is a 16.7 kDa polypeptide that has been reported to form higher molecular weight aggregates and is encoded by the *accB* gene (Li and Cronan, 1992a; Nenortas and Beckett, 1996). The carboxyltransferase component is an  $\alpha_2\beta_2$  heterotetramer with a 35.1 kDa  $\alpha$ -subunit encoded by *accA* and a 33.2 kDa  $\beta$ -subunit encoded by *accD* (Li and Cronan, 1992b).

Acetyl-CoA carboxylase (like all biotin-dependent carboxylases) is considered to have a non-classical or two-site ping-pong kinetic mechanism, which means the active sites for biotin carboxylase and carboxyltransferase are separate and do not interact or communicate with one another (Cook and Cleland, 2007). This type of kinetic mechanism is consistent with the fact that when either the carboxylase or transferase components of *E. coli* acetyl-CoA carboxylase are isolated, they retain their enzymatic activity (Guchhait *et al.*, 1974). This observation has led to two hypotheses for the mechanism of action for the enzyme. One theory posits that after BCCP

is carboxylated by biotin carboxylase, it dissociates and undergoes diffusion until it binds to carboxyltransferase. Alternatively, the second theory suggests that all the components of acetyl-CoA carboxylase form a complex where the flexible region of BCCP alternates between the active sites of biotin carboxylase and carboxyltransferase (Cronan and Waldrop, 2002). The latter mechanism involves extensive protein-protein interactions (PPI). Since PPI are well known to play significant roles in the regulation of many cellular processes such as binding of hormones to cell surface receptors, signal transduction, and allosteric regulation of enzymes, PPI may also play a role in the regulation of acetyl-CoA carboxylase activity (Ozbabacan *et al.*, 2011; Perkins *et al.*, 2010).

Understanding the regulation of bacterial acetyl-CoA carboxylase activity has become more pressing because of recent findings. It was recently proposed that during stationary phase when acetyl-CoA levels are low, carboxyltransferase binds to the mRNA coding for the  $\alpha$ - and  $\beta$ -subunits of the enzyme resulting in inhibition of both catalysis and translation of the mRNA. When acetyl-CoA levels are high, such as in the log phase of *E. coli* growth, acetyl-CoA competes with the mRNA coding for the  $\alpha$ - and  $\beta$ -subunits of carboxyltransferase thereby allowing catalysis and translation to continue (Meades *et al.*, 2010). Thus, carboxyltransferase acts as a “dimmer” switch to regulate its own enzymatic activity and gene expression at the level of translation.

This leaves the question, how is the activity of biotin carboxylase regulated when carboxyltransferase is bound to its mRNA? Most importantly, does biotin carboxylase continuously carboxylate biotin and hydrolyze ATP or is the activity decreased when carboxyltransferase is sequestered away bound to its mRNA? In this report, these questions are answered by demonstrating that all of the components of acetyl-CoA carboxylase form a

multiprotein complex where the protein-protein interactions play an integral part in regulating the activity of the enzyme.

## **Experimental Procedures**

### Reagents

Amicon Ultra Centrifugal Filters MW cutoff 10,000 were from Millipore. M280 streptavidin Dynabeads were from Invitrogen. Bradford protein assay reagent, Opti-4CN substrate kit, gel filtration standards, goat anti-mouse IgG (H+L)-HRP conjugate, and avidin-HRP conjugate were purchased from Bio-Rad. Expression vectors, Perfect Protein Marker, Nova Blue competent cells, and BL21(DE3) competent cells were obtained from Novagen. Kanamycin, streptomycin, IPTG, and PINKstain Protein Ladder were from Gold Biotechnology. Restriction endonucleases, T4 DNA ligase, and dNTPs were purchased from New England Biolabs. Pfu UltraII fusion was from Agilent. FLAG octapeptide was synthesized by Chi Scientific. BioTrace PVDF Membrane was from PALL Life Sciences. THE His-Tag Antibody Anti-His monoclonal antibody from mouse was purchased from GenScript. Casamino acids were from Lab Scientific. Deuterium oxide was from Acros. All primers (Table 2.1) were synthesized by Eurofins MWG Operon. HiLoad 16/60 Superdex 200 prepgrade gel filtration column was from Amersham Biosciences, while HisTrap HP Ni Sepharose columns were from GE Healthcare. All other reagents were from Sigma/Aldrich.

The 2-aminooxazole dibenzylamide inhibitor of biotin carboxylase was synthesized according to Mochalkin *et al.* (2009). The carboxyltransferase inhibitor, andrimid was a gift from Pfizer. To measure the deuterium kinetic isotope effect on acetyl-CoA carboxylase, D<sub>3</sub>-acetyl-CoA was synthesized by a modification of the procedure by Simon and Shemin (1953). First, in

a 25 mL flask, 40 mg of the trilithium salt of coenzyme A was dissolved in 3 mL of 1M triethylammonium bicarbonate. Next, 5 g of D<sub>6</sub>-acetic anhydride was added to the coenzyme A solution dropwise with mixing. Once all the D<sub>6</sub>-acetic anhydride was added, the mixture was left on ice for 45 min, with periodic mixing. The reaction mixture was then allowed to warm to room temperature over the course of an hour. Then 1 mL of water and 3 drops of glacial acetic acid were added to the solution. The mixture stood at room temperature overnight. The following day, the reaction was lyophilized to dryness, and the powder was resuspended in water. The concentration of D<sub>3</sub>-acetyl-CoA was determined spectrophotometrically at 260 nm using the extinction coefficient of 16,800 M<sup>-1</sup> cm<sup>-1</sup> for coenzyme A.

Primer	DNA sequence*	Plasmid	Enzyme
FlagBC5	(5' -CTTCTTCATATGGATTATAAA <b>GACGATGACGATA</b> AAAATGCTGGATAAAAATTGTTATT)	pAEP4	FLAG-BC
FlagBC3	(5' -CTTCTTTTAATTAATTATTTTCCTGAAGACCGAG)		
BCCP5	(5' -CTTCTTCATATGGATATTCGTAAGATTAATAAAA)	pAEP3	BCCP-BC
BCCP3	(5' -CTTCTTGGATCCACCTCCTTACTCGATGACGACCAGCGGCTC)		
BC5	(5' -CTTCTTGGATCCGATGCTGGATAAAAATTGTTATTGCC)	pAEP7	
BC3	(5' -CTTCTTCTCGAGTATTTTCCTGAAGAACGAG)		
FlagCTa5	(5' -CTTCTTCCATGGACTACAA <b>GACGATGACGATA</b> AAAATGAGTCTGAATTCCTTGAT)	pMEP1	FLAG-CT
FlagCTb3	(5' -CTTCTTGGATCCTCAGGCCTCAGGTTCTTGATC)		

\* The underlined nucleotides indicate a restriction endonuclease cut site. The bold nucleotides indicate a FLAG-tag coding sequence.

### Expression Vector Construction

The plasmid coding for biotin carboxylase with an N-terminal FLAG-tag (FLAG-BC) was produced by amplifying the *accC* gene using the primers FlagBC5 and FlagBC3 (Table 2.1) and pGLW1 as a template (Blanchard *et al.*, 1999b). The primers allowed for the introduction of both an *Nde*I site and the sequence coding for the FLAG-tag on the 5' end of the gene along with

a *PacI* site on the 3' end. The PCR product was cut with *NdeI* and *PacI* and ligated into pETDuet-1 cut with the same enzymes to create pAEP4.

The plasmid coding for carboxyltransferase with an N-terminal FLAG-tag on the  $\alpha$ -subunit (FLAG-CT) was constructed by amplifying the *accA* and *accD* genes using pCZB3 as a template. The plasmid pCZB3 contains the genes coding for the  $\alpha$ - and  $\beta$ -subunits of carboxyltransferase in a minioperon (Blanchard and Waldrop, 1998). Primers FlagCTa5 and FlagCTb3 (Table 2.1) introduced both an *NcoI* site and the sequence coding for the FLAG-tag on the 5' end of the *accA* gene and a *BamHI* site on the 3' end of the *accD* gene. The PCR product was cut with *NcoI* and *BamHI* and ligated into pCDFDuet-1 cut with the same enzymes to create pMEP1.

A minioperon of the *accB* and *accC* genes was constructed to generate the biotin carboxyl carrier protein – biotin carboxylase complex (BCCP-BC). The BCCP5 and BCCP3 primers were used to amplify the BCCP gene (*accB*) using pYML11 as a template. The plasmid pYML11 contains the gene for *E. coli* BCCP in pGEM7 between the *EcoRI* and *BamHI* cut sites. The primers introduced an *NdeI* site on the 5' end of the gene, and both the sequence 5' – AAGGAG-3', which encoded a ribosomal binding site, and a *BamHI* site were added to the 3' end of the gene. The PCR product was cut with *NdeI* and *BamHI* and ligated into pET28b(+) cut with the same enzymes to create pAEP3. The *accC* gene was amplified from pGLW1 using the primers BC5 and BC3 to introduce a 5' *BamHI* site and a 3' *XhoI* site. The resulting PCR product was cut with *BamHI* and *XhoI* and ligated into pAEP3 cut with the same enzymes to create pAEP7. This yielded a plasmid with an *accBC* minioperon containing a 13 bp intercistronic region that expressed an N-terminal His-tagged BCCP and biotin carboxylase.



## Protein Expression in *E. coli*

Biotin carboxylase with a His-tag on the N-terminus (Blanchard *et al.*, 1999b) and carboxyltransferase with a His-tag on the N-terminus of the  $\alpha$ -subunit (Blanchard and Waldrop, 1998) was expressed and purified as previously described. Biotinylated BCCP with a His-tag on the N-terminus was a gift from Pfizer.

FLAG-BC was produced by transforming *E. coli* strain BL21(DE3) with pAEP4 and then using a freshly transformed colony to inoculate either 0.5 liters of LB medium in a 2 liter flask or 1 liter of LB medium in a 2.8 liter Fernbach flask. The LB medium also contained 50  $\mu$ g/mL of ampicillin. The cells were incubated at 37 °C with shaking until mid-log phase. Then 250  $\mu$ M IPTG was added to the culture, which was incubated for another 2.5-3.5 h. The cells were harvested by centrifugation and frozen at -20 °C.

FLAG-CT was produced by transforming *E. coli* strain BL21(DE3) with pMEP1 and then using a freshly transformed colony to inoculate either 0.5 liters of LB medium in a two liter flask or 1 liter of LB medium in a 2.8 liter Fernbach flask containing either 10 mL glycerol or 1 g casamino acids. The LB medium also contained 50  $\mu$ g/mL of streptomycin. The flasks were incubated at 37 °C with shaking until mid-log phase and then allowed to stand and cool to room temperature. After the flasks had cooled, one of the following were added to induce expression of the gene coding for FLAG-CT: 250 $\mu$ M IPTG, 1 g lactose, or both 250  $\mu$ M IPTG and 1 g lactose. After the addition of IPTG or lactose, flasks were either incubated with shaking for 2.5-3.5 h at room temperature or they were allowed to stand at room temperature overnight. The cells were harvested by centrifugation and frozen at -20 °C. A typical preparation would involve several flasks with different various combinations of inducing agent and incubation conditions.

Overexpression of holo-acetyl-CoA carboxylase was accomplished with a two-plasmid system where plasmids pAEP7 and pMEP1 were both transformed into *E. coli* strain BL21(DE3). Overexpression was achieved with the same procedure as described above for FLAG-CT except that the LB medium contained 50 µg/mL of streptomycin and 30 µg/mL of kanamycin.

### Protein Purification

The first step in protein purification was affinity chromatography using either a nickel-based resin for proteins with a His-tag or an ANTI-FLAG M2 gel for proteins with a FLAG-tag. For proteins with a His-tag, the cell pellets were suspended in equilibration/wash buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 15 mM imidazole, pH 7.8) and then were lysed by sonication and passage through a French press. Nucleic acids were removed by the addition of DNase. The cell lysate was centrifuged at 34540 g for 1 h at 4 °C. The supernatant was loaded onto a nickel affinity column pre-equilibrated with equilibration/wash buffer and then washed with ten column volumes of the same buffer. Protein was eluted using a segmented gradient starting with eight column volumes of 0-10% elution buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 250 mM imidazole, pH 7.8), followed by 12 column volumes of 10-100% elution buffer and finally eight column volumes of 100% elution buffer. For proteins with a FLAG-tag, the cell pellets were suspended in TBS wash (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and were lysed in the same manner as for the His-tagged proteins. After centrifugation, the supernatant was loaded onto a column containing ANTI-FLAG M2 affinity gel, and protein was eluted according to the protocol provided by the manufacturer (Sigma) of the ANTI-FLAG M2 affinity resin.

Protein from either the nickel affinity or anti-FLAG columns was further purified by size exclusion chromatography. Protein eluted from either affinity column was dialyzed for 12 h against the following buffer: 134 mM KCl, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.8. The dialysate was then dialyzed for 12 h against 10 mM Hepes, 500 mM KCl, pH 7.8 (referred to hereafter as 2<sup>nd</sup> dialysis), and then concentrated using Amicon Centrifugal Filters and loaded onto a Hi-Load 16/60 Superdex 200 column pre-equilibrated with 2<sup>nd</sup> dialysis. Fractions containing pure protein were concentrated and stored at -80 °C.

#### Pull-down Assays

Assays targeting biotinylated-BCCP utilized M-280 streptavidin Dynabeads. A 100 µL suspension of beads was washed three times with 100 µL PBS buffer (2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) as described in the manufacturer's protocol. Then 50 µL of 77.8 µM BCCP was added to the beads and the beads were pelleted using a neodymium magnet. The beads were resuspended by gently shaking at 25 °C for 1 h and then pelleted again and washed once with PBS buffer. A 450 µL solution of 1.67 mM AMPPNP, 0.7 µM CT, 0.94 µM BC, 3.2 mM acetyl-CoA, 22.2 mM MgCl<sub>2</sub>, 22.2 mM potassium bicarbonate, 111.2 mM Hepes pH 7.8, and 22.2 mM biotin was added to the pelleted beads, which were resuspended by mixing at 25 °C for 45 min. The beads were pelleted with a neodymium magnet and the supernatant was removed. The pellet was washed four times with 200 µL, 150 µL, 100 µL and 90 µL of PBS, and 0.1% Tween-20 (PBST). After the last wash, the pellet was resuspended in 10 µL SDS-PAGE buffer and 20 µL PBST and incubated at 80 °C for 15 min. The beads were pelleted with a neodymium magnet, and the supernatant was removed for Western blot analysis.

For pull-down assays targeting FLAG-BC, ANTI-FLAG M2 affinity resin was washed three times with 190  $\mu\text{L}$  TBS. A solution of 55  $\mu\text{L}$  TBS and 55  $\mu\text{L}$  2<sup>nd</sup> dialysis containing 11.6  $\mu\text{M}$  FLAG-BC was added to the ANTI-FLAG M2 resin and mixed at 25  $^{\circ}\text{C}$  for 1 h. After centrifugation, the supernatant was removed, and a 450  $\mu\text{L}$  solution containing 1.7 mM AMPPNP, 0.9  $\mu\text{M}$  CT, 6.9  $\mu\text{M}$  BCCP, 3.2 mM acetyl-CoA, 22.2 mM  $\text{MgCl}_2$ , 22.2 mM potassium bicarbonate and 111.2 mM Hepes pH 7.8 was added and mixed at 25  $^{\circ}\text{C}$  for 1.5 h. The pellet was washed four times with 200  $\mu\text{L}$ , 150  $\mu\text{L}$ , 100  $\mu\text{L}$  and 90  $\mu\text{L}$  of TBS. After the last wash, the pellet was resuspended in 10  $\mu\text{L}$  SDS-PAGE buffer and 20  $\mu\text{L}$  of 500  $\mu\text{g}/\text{mL}$  FLAG peptide and incubated at 80  $^{\circ}\text{C}$  for 15 min. After centrifugation, the supernatant was removed for Western blot analysis. Pull-down assays targeting FLAG-CT were done exactly as described for the FLAG-BC pull-down assays with two exceptions. First, 55  $\mu\text{L}$  of a 22  $\mu\text{M}$  FLAG-CT solution was added to the washed resin instead of 55  $\mu\text{L}$  of an 11.6  $\mu\text{M}$  FLAG-BC solution. Second, the supernatant was removed after 40 min of mixing, and a 445  $\mu\text{L}$  solution of 1.7 mM AMPPNP, 0.95  $\mu\text{M}$  BC, 4.0  $\mu\text{M}$  BCCP, 3.3 mM acetyl-CoA, 22.5 mM  $\text{MgCl}_2$ , 22.5 mM potassium bicarbonate and 112.5 mM Hepes pH 7.8 was added and mixed at 25  $^{\circ}\text{C}$  for 1 h.

#### Western Blot Analysis

Samples were run on a SDS-PAGE gel and transferred to a PVDF membrane in transfer buffer (39 mM glycine, 48 mM Tris, 20% methanol, 0.04% SDS) for 15 h at 60 V. PVDF membrane with transferred PINKstain Protein Ladder was washed in TBST (136.9 mM NaCl, 2.68 mM KCl, 24.8 mM Tris, pH 7.4 with HCl, 0.1% Tween-20) gently shaking at 25  $^{\circ}\text{C}$  for 20 min. The membrane was then blocked with 18 mL of TBST with 5% dry milk gently shaking at 25  $^{\circ}\text{C}$  for 1 h. Then a 1:3000 ratio of 1  $\mu\text{g}/\mu\text{L}$  Anti-His monoclonal antibody and anti-FLAG antibody from mouse were added to the TBST with 5% dry milk and incubated at 25  $^{\circ}\text{C}$  gently

shaking for 1 h. The membrane was washed three times in 20 mL TBST gently shaking at 25 °C for 10 min each time and then incubated in 18 mL TBST with 5% dry milk with a 1:4800 ratio of 1 µg/µL of goat anti-mouse HRP conjugate antibody while gently shaking at 25 °C for 1 h. After three more 10 min washes in TBST, the membrane was exposed to the Opti-4CN substrate for 15 min according to the instructions provided by the manufacturer Bio-Rad.

### Kinetic Assays

The activity of biotin carboxylase and holo-acetyl-CoA carboxylase was determined spectrophotometrically by measuring the production of ADP using pyruvate kinase and lactate dehydrogenase and following the oxidation of NADH at 340 nm. Each reaction contained 17.5 units of lactate dehydrogenase, 10.5 units of pyruvate kinase, 0.5 mM phosphoenolpyruvate, 0.32 mM NADH, 20 mM MgCl<sub>2</sub>, 15 mM potassium bicarbonate and 100 mM HEPES at pH 7.8. The activity of carboxyltransferase was measured as previously described (Blanchard and Waldrop, 1998). All reactions were carried out in a total of 0.5 mL in a 1 cm path-length quartz cuvette. All reactions were initiated by addition of enzyme and were maintained at 25 °C via a circulating water bath. Spectrophotometric data was collected using an Uvikon 810 spectrophotometer interfaced to a PC with a data acquisition program.

### Kinetic Analysis

Data were analyzed by non-linear regression using the computer programs of Cleland (Cleland, 1979). Initial velocities obtained by varying one of the substrates (A) were fitted to Equation 1 to determine the maximal velocity (V) and the Michaelis constant (K<sub>m</sub>).

$$v = \frac{VA}{(K_m + A)} \quad (\text{Eq. 1})$$

When one substrate was varied at different fixed levels of another substrate, data were fitted to Equation 2 or Equation 3, describing sequential or ping-pong initial velocity patterns, respectively. In these equations,  $v$  is the experimentally determined velocity,  $V$  is the maximal velocity,  $K_a$  and  $K_b$  are the respective Michaelis constants for substrates A and B, and  $K_{ia}$  is the dissociation constant of A.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (\text{Eq. 2})$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (\text{Eq. 3})$$

Data describing noncompetitive inhibition were fitted to Equation 4, where  $V$  is the maximal velocity,  $K_m$  is the Michaelis constant,  $I$  is the concentration of inhibitor, and  $K_{is}$  and  $K_{ii}$  are the slope and intercept inhibition constants, respectively.

$$v = \frac{VA}{K_m \left(1 + \frac{I}{K_{is}}\right) + A \left(1 + \frac{I}{K_{ii}}\right)} \quad (\text{Eq. 4})$$

Multiple inhibition data were fitted to Equation 5, where  $K_L$  and  $K_J$  are the apparent dissociation constants for the two inhibitors and  $\alpha$  is a measure of the degree of interaction of the two inhibitors.

$$v = \frac{V}{\left(1 + \frac{L}{K_L} + \frac{J}{K_J} + \frac{LJ}{\alpha K_L K_J}\right)} \quad (\text{Eq. 5})$$

## Results

Bacterial acetyl-CoA carboxylase, and the *E. coli* form in particular, has been the model system for studying biotin-dependent enzymes for two reasons. First, the carboxylase and transferase components retain their activity when isolated, and second, both biotin carboxylase and carboxyltransferase will utilize free biotin (i.e. biotin not covalently attached to BCCP) as a substrate thereby simplifying kinetic analysis (Guchhait *et al.*, 1974). While the use of free biotin has allowed for detailed kinetic and mechanistic studies on biotin carboxylase and carboxyltransferase, the fact remains that *in vivo* biotinylated BCCP, not free biotin, is the substrate for these two enzymes (Cronan and Waldrop, 2002). However, biotinylated BCCP turns out not to be a very good substrate for biotin carboxylase (Figure 2.1). In fact, the rate of ATP hydrolysis by biotin carboxylase is low even when all of the components of acetyl-CoA carboxylase are present (Figure 2.1). Not until acetyl-CoA is added is a dramatic increase in the rate of ATP hydrolysis detected (Figure 2.1). This phenomenon where biotin carboxylase only hydrolyzes ATP in the presence of BCCP, carboxyltransferase, and acetyl-CoA was first noted by Soriano *et al.* (2006) and Alves *et al.* (2011) when these groups were developing high-throughput screening assays for bacterial acetyl-CoA carboxylase. The results in Figure 2.1 suggest there is communication between the active sites of biotin carboxylase and carboxyltransferase and by inference implies that all of the components form a higher order protein complex. The active site communication is an example of substrate synergism where enzyme activity is regulated by only allowing ATP to be utilized when acetyl-CoA is present thereby preventing wasteful hydrolysis of ATP.

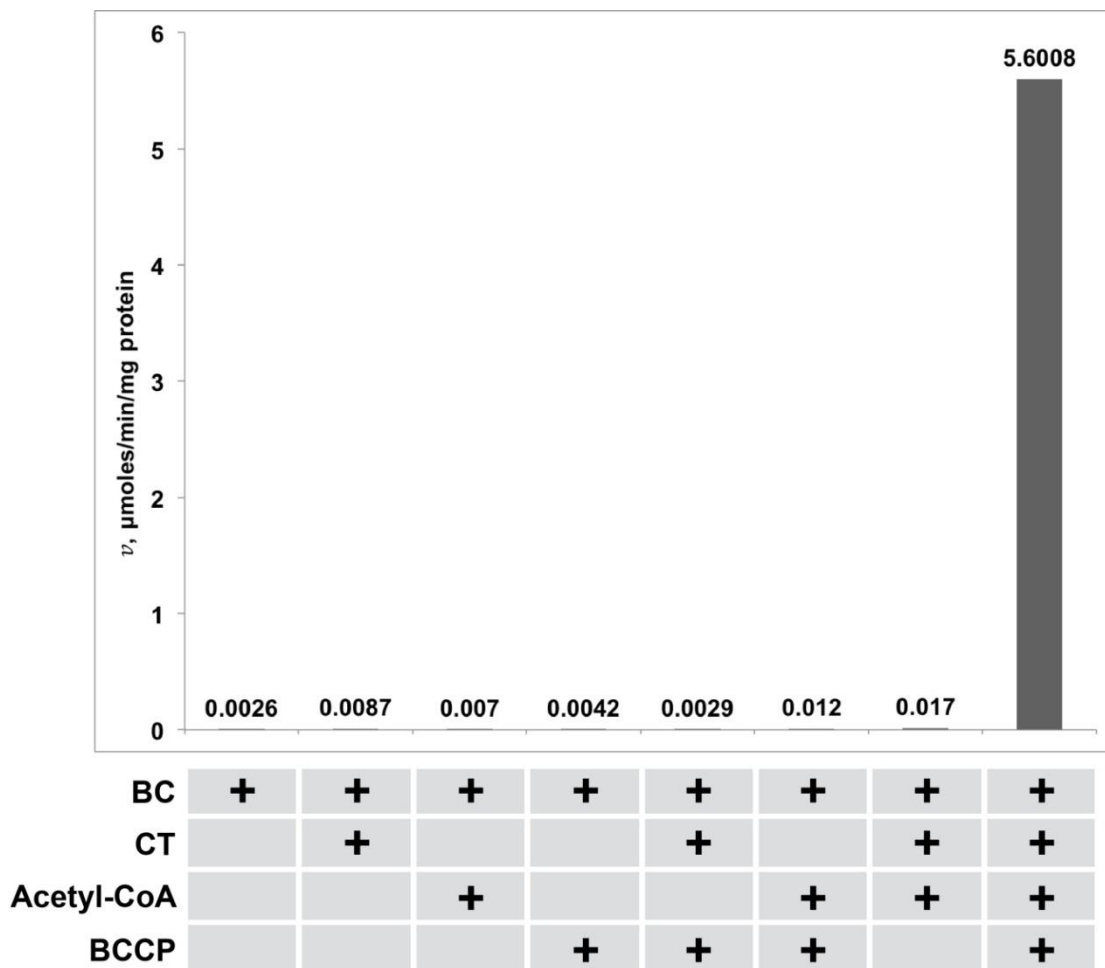


Figure 2.1 The effect of acetyl-CoA and the different components of acetyl-CoA carboxylase on the specific activity of biotin carboxylase. The specific activities were calculated based on the amount (16.1  $\mu\text{g}$ ) of BC in each reaction. Numbers above the bar indicate the specific activity and a (+) symbol below the bar indicates the presence of either 0.32  $\mu\text{M}$  BC, 0.24  $\mu\text{M}$  CT, 2  $\mu\text{M}$  BCCP, and/or 423  $\mu\text{M}$  acetyl-CoA. ATP and bicarbonate were held constant at 0.4 mM and 3.25 mM, respectively. All reactions were initiated by the addition of BC.

Two distinct experimental approaches were used to address the active site communication and whether complex formation is necessary for the regulation of acetyl-CoA carboxylase activity. The first approach involved kinetic characterization of acetyl-CoA carboxylase, while the second approach used pull-down/co-immunoprecipitation assays to detect complex formation both *in vitro* and *in vivo*.



## Kinetic Analysis

Any kinetic analyses of acetyl-CoA carboxylase must start by noting that the enzyme is considered to be a non-classical or two-site ping-pong enzyme (Figure 2.2). If acetyl-CoA carboxylase is indeed a two-site ping-pong (a.k.a. double displacement) enzyme, then each of the two half-reactions are expected to exhibit activity in the absence of the substrate for the other half-reaction. In the case of acetyl-CoA carboxylase, one would expect ATP hydrolysis in the absence of acetyl-CoA. However, as has already been pointed out, that does not occur (Figure 2.1). Conversely, when carboxyltransferase is assayed in the non-physiological direction with BCCP and malonyl-CoA as substrate, no activity could be detected in the absence of ATP (data not shown). This lack of reversibility is in stark contrast to isolated carboxyltransferase which is routinely assayed in the reverse direction (Blanchard and Waldrop, 1998). Thus, not only do the two half-reactions not exhibit independent activity as expected of a ping-pong enzyme, but also the fact that the reaction is irreversible in acetyl-CoA carboxylase but not in isolated carboxyltransferase strongly suggests the individual components form a complex.

## Isotope Effects

In the second half-reaction, the carboxyl group on carboxybiotin is transferred to acetyl-CoA. For the carboxylation reaction to occur, a proton must be removed from the methyl group of acetyl-CoA. This allows the kinetic isotope effect for substituting deuterium for hydrogen in the methyl group of acetyl-CoA to be determined. If acetyl-CoA carboxylase has a conventional ping-pong kinetic mechanism, then the isotope effect when ATP is varied,  $^D(V/K_{ATP})$ , should be unity because the first half-reaction catalyzed by biotin carboxylase does not include the isotope sensitive step. The  $^D(V/K_{ATP}) = 1.62 \pm 0.01$  while the isotope effect on the maximal velocity  $^DV$

$= 1.8 \pm 0.2$ . The lack of unity for  $^D(V/K_{ATP})$  is inconsistent with a strict ping-pong mechanism and suggests an interaction between the two half-reactions.

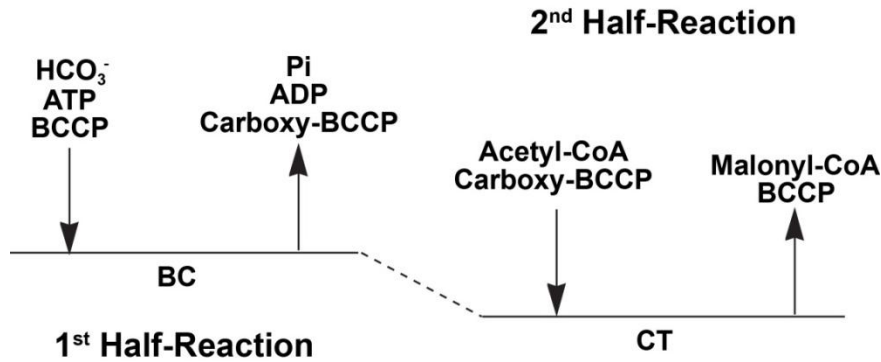


Figure 2.2 Kinetic mechanism of acetyl-CoA carboxylase as a two-site ping-pong mechanism.

The kinetic isotope effects also provide insight into the relative rates of the two half-reactions. For instance, the fact that the isotope effect on the maximal velocity ( $^D V$ ) is not equal to the intrinsic isotope effect ( $\sim 6.2$ )<sup>1</sup> indicates that the second half-reaction catalyzed by carboxyltransferase is not completely rate-limiting. Moreover, because the  $^D V$  and  $^D(V/K_{\text{acetyl-CoA}})$  are, within error, equal (the  $^D(V/K_{\text{acetyl-CoA}}) = 2.1 \pm 0.2$ ), the first half-reaction catalyzed by biotin carboxylase is not completely rate-limiting, otherwise the  $^D V$  would have been unity since the isotope sensitive step is part of the second half-reaction. Thus, the isotope effects suggest that both half-reactions are partly rate-limiting.

### Initial Velocity Patterns

The initial velocity pattern for an enzyme with a ping-pong kinetic mechanism is expected to show a set of parallel lines in a double reciprocal plot. In other words, when varying

<sup>1</sup> The removal of a proton from the methyl group of acetyl-CoA is analogous to a keto-enol conversion. Keeffe and Kresge (1996) measured the deuterium isotope effect on the enolization of isobutyrophenone as 6.2. Therefore, the intrinsic isotope effect for substituting deuterium for hydrogen at the methyl group of acetyl-CoA is estimated to be about 6.2.

the concentration of the substrate for a particular half-reaction, the slope in a double reciprocal plot is not a function of the concentration of the substrate for the other half-reaction. When ATP was varied at different fixed levels of acetyl-CoA (Figure 2.3 A), the data fit best to Equation 3, which describes a set of parallel lines expected for a ping-pong kinetic mechanism.

In contrast, when acetyl-CoA was varied at different fixed levels of ATP (Figure 2.3 B), a set of intersecting lines was observed. The data in Figure 2.3 B were fitted to both Equation 2 and Equation 3. The data fit best to Equation 2, describing a sequential initial velocity pattern, based on the fact that the average least squares of the residuals were the lowest for Equation 2. The intersecting initial velocity pattern in Figure 2.3 B indicates that all substrates (in this case ATP and acetyl-CoA) must be on the enzyme for catalysis to occur. A change in slope with increasing ATP means that even at very low acetyl-CoA, ATP affects the reaction rate. This suggests communication between the biotin carboxylase and carboxyltransferase active sites.

The two initial velocity patterns are seemingly contradictory in that when ATP is varied at fixed levels of acetyl-CoA a ping-pong mechanism is indicated while the intersecting pattern when acetyl-CoA is varied at fixed levels of ATP is characteristic of a sequential mechanism. These two contradictory observations can be reconciled by noting that an initial velocity pattern for a sequential mechanism can resemble the pattern for a ping-pong mechanism. If the  $K_{ia}$  parameter in Equation 2 (which is the dissociation constant for A) was much less than the Michaelis constant for A ( $K_a$ ) then Equation 2 would be approximately equal to Equation 3 and the initial velocity would appear as a set of parallel lines (Cook and Cleland, 2007). However, despite the appearance of a ping-pong kinetic mechanism the true mechanism is in fact sequential.

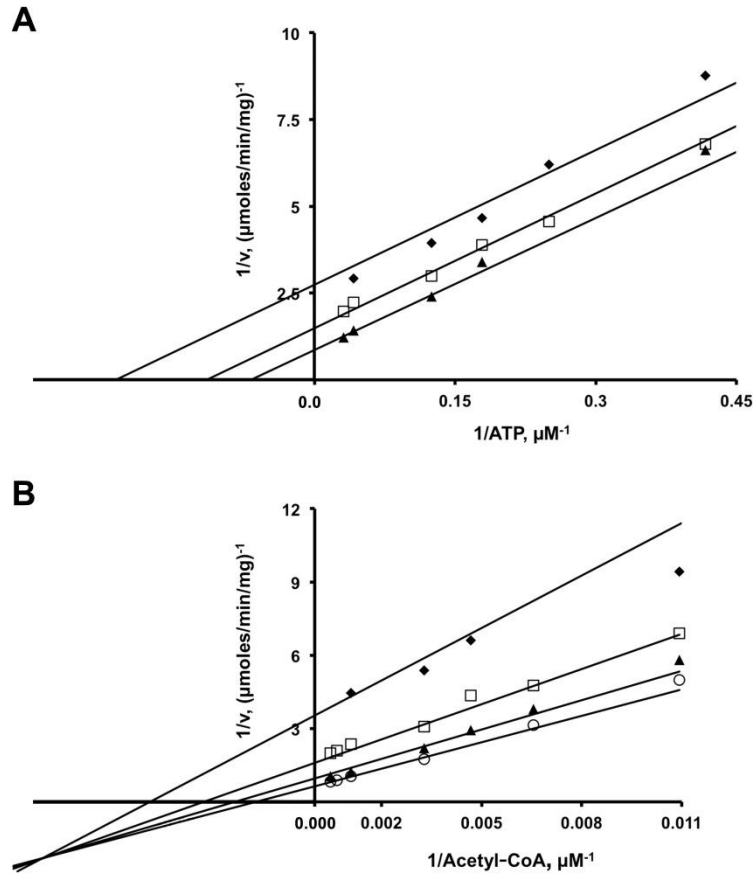


Figure 2.3 Initial velocity patterns of acetyl-CoA carboxylase. **A**) initial velocity pattern versus ATP concentration at different fixed levels of acetyl-CoA at 0.3 mM ( $\blacklozenge$ ), 0.6 mM ( $\square$ ) and 1.2 mM ( $\blacktriangle$ ). **B**) initial velocity pattern versus acetyl-CoA concentration at different fixed levels of ATP at 2  $\mu\text{M}$  ( $\blacklozenge$ ), 5  $\mu\text{M}$  ( $\square$ ), 10  $\mu\text{M}$  ( $\blacktriangle$ ) and 20  $\mu\text{M}$  ( $\circ$ ). Both assays contained 2  $\mu\text{M}$  BCCP, 0.32  $\mu\text{M}$  BC, and 0.24  $\mu\text{M}$  CT in each reaction. The points represent the observed velocities, and the lines represent the best fit of the data to equation 3 (**A**) and equation 2 (**B**).

It is important to point out that the kinetics observed here with the bacterial form of the enzyme are very similar to those observed with various forms of eukaryotic acetyl-CoA carboxylase. For instance, initial velocity patterns of acetyl-CoA carboxylase from chicken liver (Beaty and Lane, 1982), maize (Herbert *et al.*, 1996), and the human mitochondrial isoform (Kaushik *et al.*, 2009) all exhibit an intersecting initial velocity pattern when acetyl-CoA is varied at different fixed levels of ATP and when ATP is varied at different fixed levels of acetyl-CoA. The authors of each of those studies concluded that the two different active sites in the

various eukaryotic acetyl-CoA carboxylases interact or communicate with each other and that the enzyme does not have a strict ping-pong kinetic mechanism.

### Inhibition Studies

Given that acetyl-CoA carboxylase did not show an initial velocity pattern expected of a ping-pong enzyme, dead-end inhibition analyses were carried out to further probe the kinetic properties of the enzyme. Enzymes with ping-pong kinetic mechanisms would be expected to exhibit uncompetitive inhibition when the substrate for a half-reaction is varied with different fixed levels of an inhibitor that binds to the enzyme catalyzing the other half-reaction. Therefore, an inhibitor of biotin carboxylase was examined as a function of varying levels of acetyl-CoA, while an inhibitor of carboxyltransferase was examined as a function of varying levels of ATP.

The biotin carboxylase inhibitor, 2-aminooxazole dibenzylamide, was developed by Pfizer as an antibiotic and was found to be competitive with respect to ATP (Mochalkin *et al.*, 2009). The carboxyltransferase inhibitor, andrimid, is a natural product antibiotic that exhibits competitive inhibition with respect to acetyl-CoA (Freiberg *et al.*, 2004). The 2-aminooxazole dibenzylamide inhibitor of biotin carboxylase would be expected to exhibit uncompetitive inhibition with respect to acetyl-CoA if acetyl-CoA carboxylase is a strictly ping-pong mechanism. Likewise, the carboxyltransferase inhibitor, andrimid, would be expected to exhibit uncompetitive inhibition with respect to ATP. However, as shown in Figure 2.4 A and B, 2-aminooxazole dibenzylamide is a noncompetitive inhibitor versus acetyl-CoA, while andrimid is a noncompetitive inhibitor versus ATP, respectively. The noncompetitive inhibition pattern indicates that inhibitor binding at the active site of either biotin carboxylase or carboxyltransferase is affecting binding and catalysis in the other enzyme, which again implies communication between the two proteins.

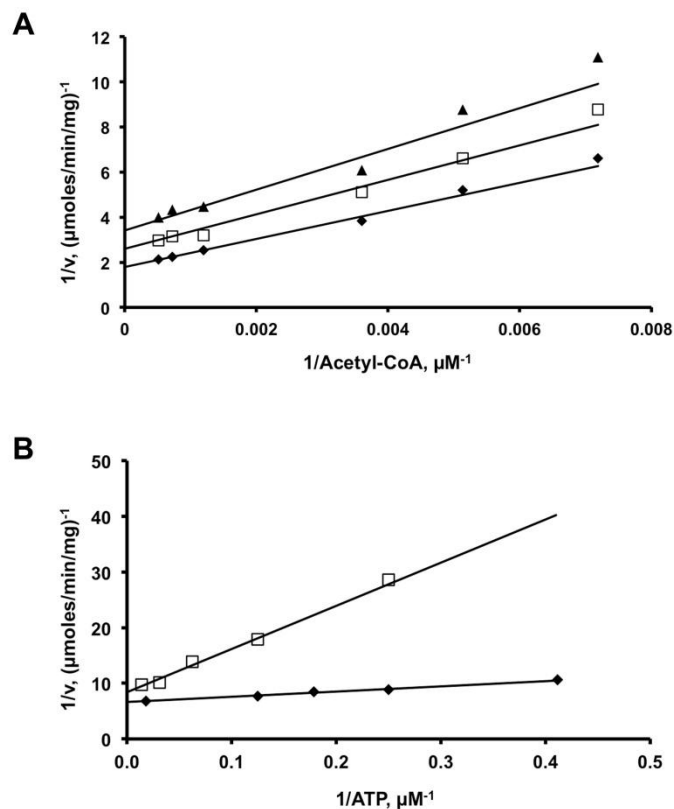


Figure 2.4 Dead-end inhibition of acetyl-CoA carboxylase. **A**) Inhibition by 2-aminooxazole dibenzylamide at 0  $\mu\text{M}$  (◆), 0.5  $\mu\text{M}$  (□), and 1  $\mu\text{M}$  (▲) while varying acetyl-CoA and holding ATP constant at 10  $\mu\text{M}$ . **B**) Inhibition by andrimid at 0 nM (◆) and 15 nM (□) while varying ATP and holding acetyl-CoA constant at 200  $\mu\text{M}$ . Both assays contained 2  $\mu\text{M}$  BCCP, 0.32  $\mu\text{M}$  BC, and 0.24  $\mu\text{M}$  CT in each reaction. The points represent the observed velocities, and the lines represent the best fit of the data to equation 4.

### Multiple Inhibition

Since inhibitor binding at one active site affected the activity of the other enzyme, multiple inhibition analysis was undertaken to determine whether inhibitor binding to the active sites of biotin carboxylase and carboxyltransferase was synergistic or antagonistic. Multiple inhibition analyses were carried out by measuring the initial velocities at increasing concentrations of one inhibitor while the second inhibitor was held constant. Initial velocities were measured again at higher levels of the second inhibitor and then plotted as 1/velocity versus

the concentration of the first inhibitor (sometimes referred to as a Yonetani-Theorell plot) (Yonetani, 1982). The substrate concentrations were held constant at subsaturating levels. For multiple inhibition analysis of acetyl-CoA carboxylase, one inhibitor was the biotin carboxylase inhibitor 2-aminooxazole dibenzylamide. For the carboxyltransferase inhibitor, the product malonyl-CoA was used instead of andrimid because malonyl-CoA is water soluble whereas andrimid is dissolved in DMSO. The levels of andrimid required for multiple inhibition studies would exceed the amount of DMSO that acetyl-CoA carboxylase can tolerate.

When 2-aminooxazole dibenzylamide was varied at different fixed levels of malonyl-CoA, an intersecting pattern was observed that indicates the two inhibitors can bind to the enzyme simultaneously (Figure 2.5). When the data were fitted to equation 5, an  $\alpha$  value of .69 was obtained. The  $\alpha$  value is an indication of the interaction of the two inhibitors. Values of  $\alpha > 1$  indicate that the binding of the two inhibitors interfere with one another, while an  $\alpha$  value of 1 indicates no interaction between the inhibitors and an  $\alpha$  value  $< 1$  indicates synergism in the binding of the inhibitors. Thus, the fact that two inhibitors, which bind to biotin carboxylase and carboxyltransferase, interact synergistically suggests an interaction between the active sites.

In summary, the steady-state kinetic analysis of acetyl-CoA carboxylase taken as a whole indicates the enzyme has some characteristics of an enzyme with a sequential kinetic mechanism. For example, all the substrates must be on the enzyme for catalysis to occur. The fact that acetyl-CoA carboxylase does not behave as a ping-pong enzyme and that the two-half reactions are interacting implies a physical association between the proteins. In order to measure directly if the proteins comprising the multifunctional acetyl-CoA carboxylase form a complex, pull-down or co-immunoprecipitation assays were employed.

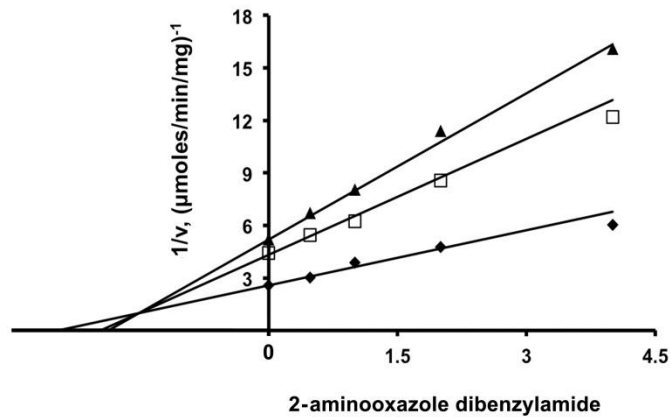


Figure 2.5 Multiple inhibition of acetyl-CoA carboxylase. Inhibition varying 2-aminooxazole dibenzylamide at different fixed levels of malonyl-CoA at 0  $\mu\text{M}$  ( $\blacklozenge$ ), 200  $\mu\text{M}$  ( $\square$ ), and 300  $\mu\text{M}$  ( $\blacktriangle$ ). ATP, acetyl-CoA, BCCP, BC, and CT were held constant at 10  $\mu\text{M}$ , 399  $\mu\text{M}$ , 2  $\mu\text{M}$ , 0.32  $\mu\text{M}$ , and 0.24  $\mu\text{M}$ , respectively. The points represent the observed velocities, and the lines represent the best fit of the data to equation 5.

### Pull-down Assays

The rationale behind the pull-down assays was simply to pull-down one of the components of acetyl-CoA carboxylase and see if the other two components are also pulled-down. Biotin carboxylase or carboxyltransferase was targeted for pull-down/co-immunoprecipitation by placing an octapeptide FLAG-tag on the N-terminus of the protein. This allows anti-FLAG binding resin (which is an antibody to the FLAG peptide) to be used to pull down any complexes that form. To target biotinylated BCCP, the protein streptavidin was used because it binds biotin very tightly ( $K_d = 10^{-15}$  M) (Green, 1963).

Pull-down assays of BCCP with streptavidin in the presence of carboxyltransferase and biotin carboxylase showed that all three components do indeed form a complex and that when BCCP was removed, only slight non-specific binding by carboxyltransferase was observed (Figure 2.6, lanes 5 and 6). Likewise, when FLAG-CT was pulled-down in the presence of biotin



carboxylase and BCCP, all three proteins were detected, and when FLAG-CT was removed, neither biotin carboxylase nor BCCP was observed (Figure 2.6, lanes 7 and 8). Finally, both carboxyltransferase and BCCP were pulled-down when FLAG-BC was targeted and were not pulled-down when FLAG-BC was absent (Figure 2.6, lanes 9 and 10). Thus, irrespective of which component of acetyl-CoA carboxylase is targeted, the other two components are also pulled-down. These data strongly suggest these proteins can form a complex, at least *in vitro*, and are consistent with the steady-state kinetic analysis.

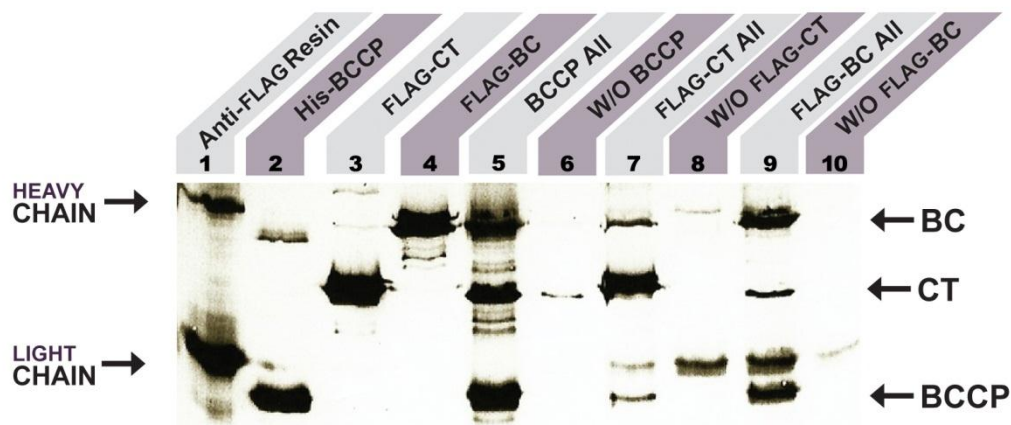


Figure 2.6 Pull-down assays targeting the individual components of acetyl-CoA carboxylase. *Lane 1*, anti-FLAG resin showing the heavy and light chains of the anti-FLAG resin antibodies. *Lanes 2-4*, input lanes of His-BCCP, FLAG-CT and FLAG-BC, respectively. *Lanes 5-6*, pull-down assay using streptavidin beads to target biotinylated BCCP in the presence of BC and CT and absence of BCCP, respectively. *Lanes 7-8*, pull-down assay using anti-FLAG resin to target FLAG-CT in the presence of BCCP and BC and absence of FLAG-CT, respectively. *Lanes 9-10*, pull-down assay using anti-FLAG resin to target FLAG-BC in the presence of BCCP and CT and absence of FLAG-BC, respectively.

In order to determine if the substrates have an effect on complex formation, pull-down assays targeting FLAG-BC were carried out in the presence or absence of acetyl-CoA and the non-hydrolyzable analog of ATP, AMPPNP. As shown in Figure 2.7, neither acetyl-CoA nor AMPPNP had any effect on complex formation. The lane containing only AMPPNP has similar band intensities within the lane, and the reduction in band intensities in comparison to the other

lanes is due to a reduced amount of resin at the end of the experiment. In addition, the mRNA coding for the  $\alpha$ -subunit of carboxyltransferase, which has been shown to bind to carboxyltransferase (Meades *et al.*, 2010), also did not have any effect on complex formation (data not shown).

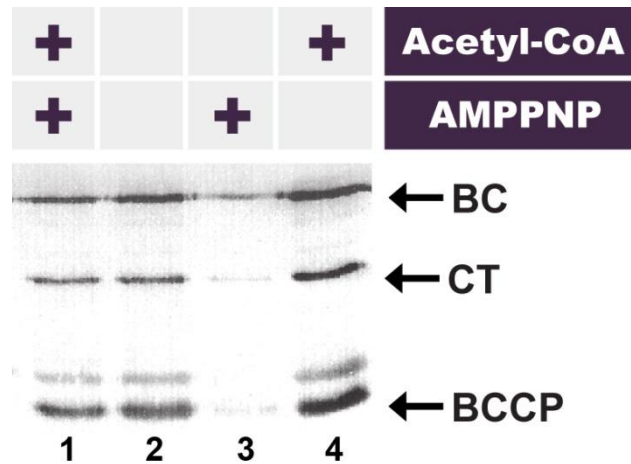


Figure 2.7 The effect of acetyl-CoA and AMPPNP on the pull-down assays targeting the individual components of acetyl-CoA carboxylase. In all lanes, FLAG-BC was targeted with anti-FLAG resin to pull-down CT and BCCP. *Lane 1*, Pull-down assay in the presence of 3.22 mM acetyl-CoA and 1.67 mM AMPPNP. *Lane 2*, Pull-down assay in the absence of acetyl-CoA and AMPPNP. *Lane 3*, Pull-down assay in the absence of acetyl-CoA and the presence of 1.67 mM AMPPNP. *Lane 4*, Pull-down assay in the presence of 3.22 mM acetyl-CoA and the absence of AMPPNP.

While the pull-down assays showed the three components of acetyl-CoA carboxylase form a complex *in vitro*, the next question was could the multiprotein complex also form *in vivo*? Initially, the bacterial two-hybrid system described by Dove *et al.* (1997) was used to detect *in vivo* interactions. This approach proved to be problematic and inconclusive, most likely because the technique involved fusing proteins to the N-termini of biotin carboxylase and carboxyltransferase. Therefore, to examine complex formation *in vivo*, a minioperon containing the genes for the  $\alpha$ - and  $\beta$ -subunits of carboxyltransferase was constructed in one plasmid, while another plasmid with a different origin of replication, contained a minioperon with the genes

coding for biotin carboxylase and an N-terminal His-tagged BCCP. Overexpression of the genes, cell lysis, and purification of the His-tagged BCCP by nickel-affinity chromatography revealed that all of the subunits of acetyl-CoA carboxylase co-purify as a single complex (Figure 2.8). The complex remained intact even after gel filtration chromatography, where the major peak of acetyl-CoA carboxylase activity eluted before the 640 kDa standard (Figure 2.9 A and B). A molecular weight > 640 kDa indicates that the multiprotein complex is not simply composed of a biotin carboxylase homodimer bound to two monomers of BCCP and a heterotetramer of carboxyltransferase which would have a molecular weight of 269 kDa. Instead, the data suggests acetyl-CoA carboxylase is a large macromolecular complex. The stoichiometry of the multiprotein complex is not known and will have to await the determination of the crystal structure of acetyl-CoA carboxylase which is underway.

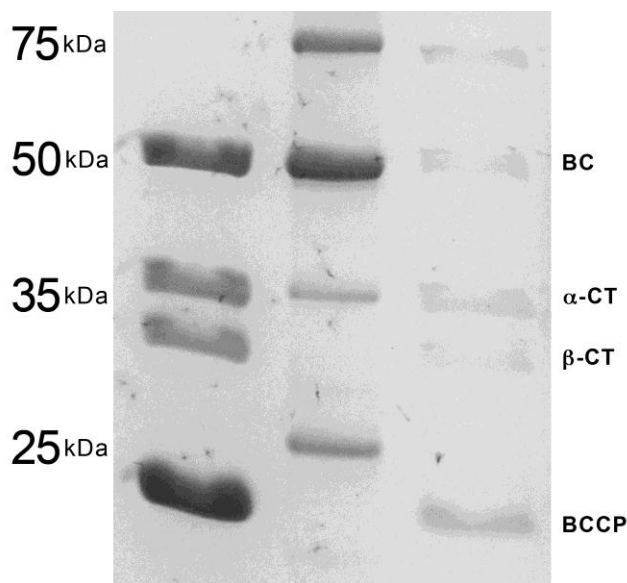


Figure 2.8 Pull-down assay of acetyl-CoA carboxylase produced *in vivo*. *Lane 1*, SDS-PAGE of ACC after purification on a nickel affinity column. *Lane 2*, molecular weight markers. *Lane 3*, SDS-PAGE of ACC after affinity purification on an anti-FLAG column.

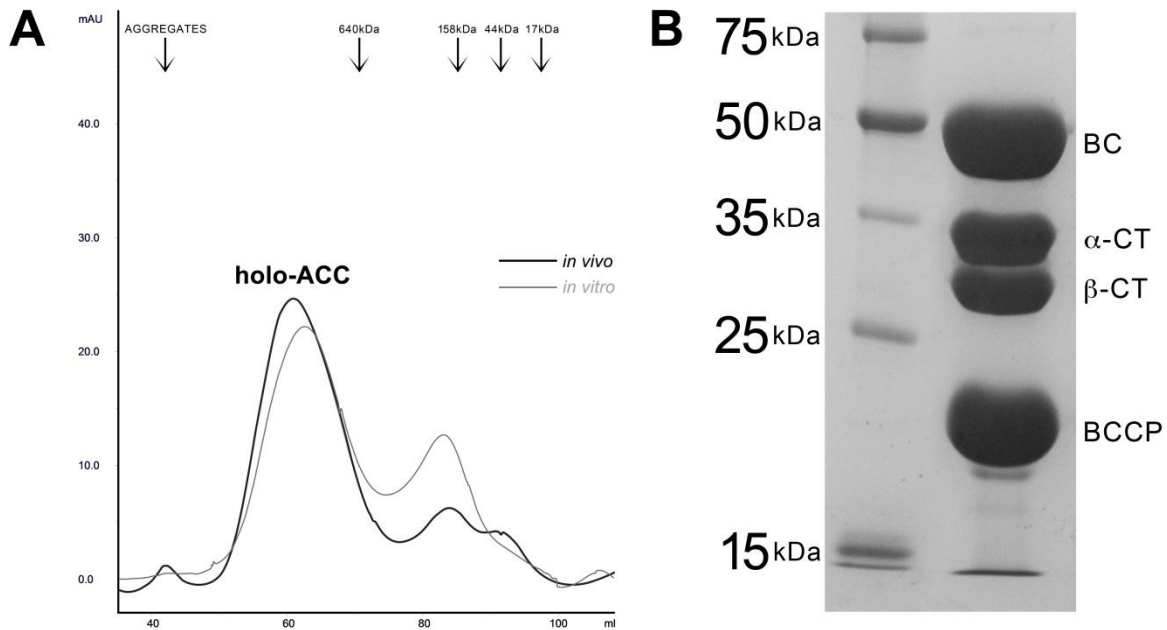


Figure 2.9 Gel filtration of acetyl-CoA carboxylase produced *in vivo* and *in vitro*. **A)** The solid black and grey lines denote the mAU of the elution off the size exclusion column for *in vivo* and *in vitro* holo-ACC respectively, and “holo-ACC” marks the peak containing ACC from the *in vivo* run, which corresponds to *Lane 2* in **B**. The arrows at the top of the figure represent which mL the molecular weight markers elute. **B)** *Lane 1*, molecular weight markers. *Lane 2*, *in vivo* ACC after purification on a nickel-affinity column followed by a size exclusion column.

In order to determine if the *in vivo* complex formation is not simply due to using His-tagged BCCP, the whole process was repeated using FLAG-CT as the targeted protein and anti-FLAG resin in place of the nickel affinity resin. The same results were obtained in that all three components co-purified on the anti-FLAG affinity column (Figure 2.8). To determine if the acetyl-CoA carboxylase complex is only stable when formed *in vivo*, purified biotin carboxylase, BCCP, and carboxyltransferase were combined and subjected to gel filtration chromatography. Just like the acetyl-CoA carboxylase that was produced *in vivo*, a peak eluting before the 640 kDa standard was observed (Figure 2.10). SDS-PAGE analysis of the major peak showed it contained the four separate proteins that comprise acetyl-CoA carboxylase (Figure 2.10). The bands corresponding to the  $\alpha$ - and  $\beta$ -subunits of CT in Figure 2.10 are slightly different from the

*in vivo* assembled acetyl-CoA carboxylase. Since the stoichiometry of the multiprotein complex is unknown, excess BCCP and BC was mixed with CT to ensure complex formation. Excess BCCP-BC forms a complex with a quaternary structure larger than 640 kDa (unpublished observations) that is outside the resolution range of the size exclusion column that would allow separation from holo-ACC and accounts for the differing band intensities in Figure 2.10.

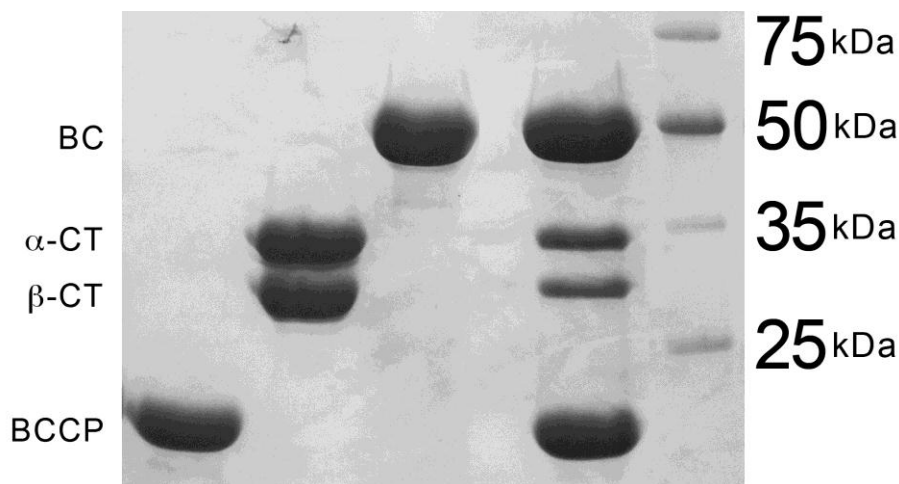


Figure 2.10 SDS-PAGE of acetyl-CoA carboxylase produced *in vitro*. Purified BC, BCCP, and CT were combined *in vitro* and then subjected to size exclusion chromatography. *Lane 1*, BCCP. *Lane 2*, FLAG-CT. *Lane 3*, BC. *Lane 4*, *in vitro* ACC after combining lanes 1-3 and purification over a size exclusion column. *Lane 5*, molecular weight markers.

Thus, all of the evidence taken together (enzyme kinetics along with the *in vitro* and *in vivo* pull-down assays) strongly argue that acetyl-CoA carboxylase forms a higher order protein structure and that formation of this multiprotein complex is necessary for regulating the enzymatic activity whereby ATP is only hydrolyzed in the presence of acetyl-CoA.

## Discussion

Protein-protein interactions play a multitude of roles in cellular and organismal functions such as signal transduction, blood coagulation, immunity, etc. The complexes involved in PPI

have been characterized as being either obligate or non-obligate (Nooren and Thornton, 2003; Ozbabacan *et al.*, 2011). Obligate PPI are distinguished from non-obligate PPI in that the different protein components cannot exist independently and that the protein interactions are irreversible (Perkins *et al.*, 2010). Acetyl-CoA carboxylase seems to fall into both categories because biotin carboxylase and carboxyltransferase can be isolated and retain their enzymatic function indicative of a non-obligate PPI. However, all the components together form a multimeric complex that appears to be stable, at least to the extent that it remains intact after size exclusion chromatography, consistent with an obligate PPI. The results in this study provide the first evidence that all the components of acetyl-CoA carboxylase form a complex, which is in direct contrast to previous reports that the multienzyme complex is labile and transient in nature (Cronan and Waldrop, 2002; Polyak *et al.*, 2012).

These seemingly contrasting observations can be reconciled by noting that while biotin carboxylase and carboxyltransferase do indeed retain their catalytic activity when isolated, that activity is only observed when free biotin is used as a substrate (Guchhait *et al.*, 1974). When biotin covalently linked to BCCP is the substrate, biotin carboxylase or carboxyltransferase have little to no activity. It is only when acetyl-CoA and all three components, biotin carboxylase, BCCP, and carboxyltransferase, are present that the enzyme exhibits full enzymatic activity. Therefore, the PPI in acetyl-CoA carboxylase could be termed obligate because the individual components do not function independently and the interactions are irreversible. The question now is: why do isolated biotin carboxylase and carboxyltransferase have activity with free biotin but not with biotin covalently linked to BCCP?

The N-terminal domain of BCCP appears to be the structural feature that impedes enzymatic activity of isolated biotin carboxylase or carboxyltransferase when BCCP is the

substrate. This conclusion is based on the observation that the biotinylated, C-terminal domain of BCCP (referred to as BCCP87 because it contains 87 amino acids) was an excellent substrate for isolated biotin carboxylase and carboxyltransferase (Chapman-Smith *et al.*, 1994). In fact, the catalytic efficiency of BCCP87 for isolated biotin carboxylase and carboxyltransferase was 8000- and 2000-fold higher than for free biotin, respectively (Blanchard *et al.*, 1999a). In other words, in the absence of the N-terminal domain, BCCP works quite well as a substrate for either biotin carboxylase or carboxyltransferase. Therefore, one possible role for the N-terminal domain of BCCP is to inhibit catalysis by biotin carboxylase, thereby preventing wasteful ATP hydrolysis in the absence of carboxyltransferase and/or acetyl-CoA.

The lack of reversibility in the reaction catalyzed by acetyl-CoA carboxylase actually supports the idea that acetyl-CoA carboxylase catalyzes the rate-determining step in fatty acid synthesis (Davis *et al.*, 2000). Specifically, the fact that acetyl-CoA carboxylase will not catalyze the reverse reaction (i.e. the conversion of malonyl-CoA into acetyl-CoA) means the first step in fatty acid synthesis is not at equilibrium, which is exactly what is wanted in a reaction that is the rate-determining step in a metabolic pathway. The consequence of the irreversibility of acetyl-CoA carboxylase is that all of the product of the reaction, malonyl-CoA, will be utilized by fatty acid synthase, and thus, the flux through fatty acid synthesis will be at least partly determined by the number of enzyme molecules. This concept fits perfectly with the model proposed by Meades *et al.* (2010) wherein the stationary phase of *E. coli* growth, carboxyltransferase acts as a “dimmer switch” by binding to the mRNA coding for the  $\alpha$ - and  $\beta$ -subunits, which inhibits translation as well as enzymatic activity. In contrast, during log phase, acetyl-CoA levels increase dramatically and compete with mRNA for binding to carboxyltransferase. This allows for substrate turnover as well as translation of mRNA to synthesize more carboxyltransferase

molecules, resulting in an increase in flux through fatty acid synthesis. Thus, acetyl-CoA, which acts as a sensor of the metabolic state of *E. coli*, regulates acetyl-CoA carboxylase, first by upregulating the synthesis of the carboxyltransferase component and second, by stimulating enzymatic activity. These two levels of regulation mediated by acetyl-CoA minimize the unnecessary hydrolysis of ATP whenever acetyl-CoA levels are low, while at the same time allowing the flux of the fatty acid synthetic pathway to be controlled by the number of enzyme molecules.

Lastly, the work described here not only examines the basic mechanism and regulation of acetyl-CoA carboxylase but it also has potential medical ramifications. Both biotin carboxylase and carboxyltransferase have been validated as new targets for antibiotic development. Compounds that inhibit biotin carboxylase (Miller *et al.*, 2009; Mochalkin *et al.*, 2009) and carboxyltransferase (Freiberg *et al.*, 2004) have been found to have antibacterial activity. These compounds inhibit biotin carboxylase and carboxyltransferase in the traditional manner, by binding in the active sites and preventing substrate binding. However, because acetyl-CoA carboxylase clearly functions as a multiprotein complex, this greatly expands the number of potential target sites beyond only the active sites to also include the sites of protein-protein interactions for the different components of acetyl-CoA carboxylase. Moreover, the multienzyme complex provides the opportunity to make a multiligand inhibitor that incorporates both biotin carboxylase and carboxyltransferase inhibitors. Not only would an acetyl-CoA carboxylase multiligand inhibitor be potentially more potent than either one of the individual ligands, but a multiligand antibacterial agent would also lessen the chance of pathogenic bacteria developing resistance (Corson *et al.*, 2008; Le Calvez *et al.*, 2009; Morphy and Rankovic, 2005; Silver, 2007).



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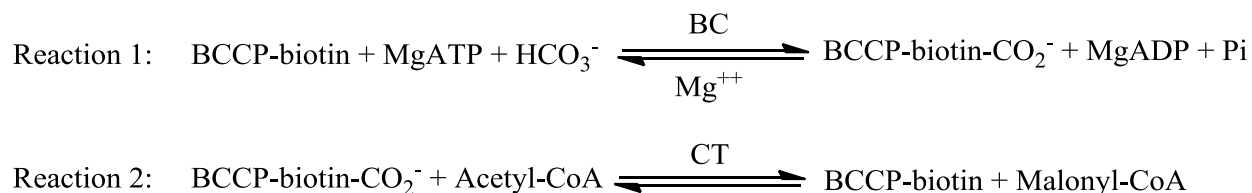
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**CHAPTER 3**  
**THE THREE-DIMENSIONAL STRUCTURE OF THE BIOTIN CARBOXYLASE-  
BIOTIN CARBOXYL CARRIER PROTEIN COMPLEX OF *E. COLI* ACETYL-COA  
CARBOXYLASE<sup>1</sup>**

**Introduction**

Biotin-dependent acetyl-coenzyme A carboxylase (ACC) catalyzes the regulated step in fatty acid synthesis in all domains of life. In *E. coli*, ACC is made up of three components: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT) (Cronan and Waldrop, 2002). The first step in the enzymatic reaction, catalyzed by BC, is the ATP dependent carboxylation of the vitamin biotin (Scheme 3.1), which is covalently attached to a specific lysine residue (Lys122) of BCCP. The second half-reaction carried out by CT is the transfer of the carboxyl group from the 1'N of carboxybiotin to acetyl-coenzyme A (acetyl-CoA) to form malonyl-CoA (Scheme 3.1).



Scheme 3.1 Acetyl-CoA carboxylase half-reactions.

The genes encoding BC (Li & Cronan, 1992a) and CT (Li & Cronan, 1992b) have been cloned, overexpressed (Blanchard *et al.*, 1999b; Blanchard and Waldrop, 1998), retain their enzymatic function in the absence of the other components, and utilize free biotin as a substrate instead of biotin linked to BCCP (Guchhait *et al.*, 1974). The structure of *E. coli* BC has been

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<sup>1</sup> *Structure*, March 14, 2013; 21(4), 650-657. The Three-Dimensional Structure of the Biotin Carboxylase-Biotin Carboxyl Carrier Protein Complex of *E. coli* Acetyl-CoA Carboxylase. Reprinted by permission of *Structure* and Elsevier.

determined in the absence of substrates (Thoden *et al.*, 2000; Waldrop *et al.*, 1994) and with ATP analogues AMPPNP and ADPCF<sub>2</sub>P (Mochalkin *et al.*, 2008) and with biotin, bicarbonate, and Mg-ADP (Chou *et al.*, 2009). The structure of CT has also been determined (Bilder *et al.*, 2006). In addition, the structure of the C-terminal domain of BCCP, which contains the biotinylated lysine, has been determined by X-ray crystallography (Athappilly and Hendrickson, 1995) and nuclear magnetic resonance (NMR) (Yao *et al.*, 1997). While the three-dimensional structures of the individual components have been of inestimable value, the fact remains that BCCP is the substrate for BC and CT, and therefore, what is lacking is a structure of either the biotin carboxyl carrier protein-biotin carboxylase (BCCP-BC) complex or the biotin carboxyl carrier protein-carboxyltransferase complex.

It was found that a stable BCCP-BC complex formed when purifying BCCP from an *E. coli* strain that coexpressed both proteins (Nenortas and Beckett, 1996), while Choi-Rhee and Cronan (2003) suggested that the stoichiometry of the protein complex was one BC homodimer bound to four BCCP molecules. Thus, since BC and BCCP form a complex, we undertook the determination of the crystal structure of that protein-protein interaction. Here we report the three-dimensional structure of the *E. coli* BCCP-BC complex to 2.49 Å resolution. The structure indicates a stoichiometry different from that previously reported (Choi-Rhee and Cronan, 2003) and reveals a quaternary structure for BC that has heretofore never been observed.

## **Experimental Procedures**

### Reagents

Amicon Ultra Centrifugal Filters MW cutoff 10,000 and Millex-HP 0.45 µM filter units were from Millipore. Bradford protein assay reagent and gel filtration standards were from Bio-

Rad. Expression vectors, Perfect Protein Markers, Nova Blue competent cells, and BL21(DE3) competent cells were obtained from Novagen. Kanamycin and IPTG were from Gold Biotechnology. Restriction endonucleases, T4 DNA ligase, and dNTPs were purchased from New England Biolabs. Pfu Ultra II fusion was from Agilent. All primers were synthesized by Eurofins MWG Operon. Highload 16/60 Superdex 200 pregrade gel filtration column was from Amersham Biosciences, TSK gel G3000SW<sub>XL</sub> column was from Tosoh Bioscience, and HisTrap HP Ni Sepharose columns were from GE Healthcare. Crystallization reagents, 24-well sitting drop trays, 0.1-0.2 mm mounted cryoloops, and CrystalCap Magnetic caps were purchased from Hampton Research. All other reagents were from Sigma/Aldrich.

#### Expression Vector Construction

In order to generate a BCCP-BC complex, a minioperon containing the *accB* and *accC* genes was constructed. The primers BCCP5 and BCCP3 (shown below) were used to amplify the *E. coli* BCCP gene (*accB*) using pYML11 as a template because it contains the gene for *E. coli* BCCP in pGEM7 between the *EcoRI* and *BamHI* cut sites. The primers introduced an *NdeI* site on the 5' end of the gene and on the 3' end of the gene a ribosomal binding site of the sequence 5'-AAGGAG-3' followed by a *BamHI* cut site. The PCR product was digested with *NdeI* and *BamHI* and ligated into pET28b(+) cut with the same enzymes to create pAEP3. The *accC* gene was amplified from pGLW1 (Blanchard *et al.*, 1999b) using the primers BC5 and BC3 (shown below) to introduce a 5' *BamHI* site and a 3' *XhoI* site. The PCR product was digested with *BamHI* and *XhoI* and ligated into pAEP3 cut with the same enzymes to create pAEP7. The final product was a plasmid containing an *accBC* minioperon with a 13 bp intercistronic region that expressed an N-terminal HIS-tagged BCCP and BC.

Primer	DNA Sequence
BCCP5	(5' -CTTCTTCATATGGATATTCGTAAGATTAAAAA- 3')
BCCP3	(5' -CTTCTTGGATCCACCTCCTTACTCGATGACGACCAGCGGCTC- 3')
BC5	(5' -CTTCTTGGATCCGATGCTGGATAAAATTGTTATTGCC- 3')
BC3	(5' -CTTCTTCTCGAGTTATTTTTCTGAAGAACGAG- 3')

### Protein Expression in *E. coli*

The BCCP-BC complex or BC was expressed by transforming *E. coli* strain BL21(DE3) with pAEP7 or pGLW2 (Blanchard *et al.*, 1999b), respectively. A freshly transformed colony was then used to inoculate either 0.5 L of LB medium in a 2 L flask or 1 L of LB medium in a 2.8 L Fernbach flask. Each flask contained 30 µg/mL kanamycin. The cultures were incubated at 37 °C with shaking until reaching mid-log phase or a 0.6 O.D. at which point they were allowed to cool for 15 min (at 25 °C) then induced with 250 µM IPTG. After a 2.5-3.5 h induction with shaking at 25 °C, the cells were harvested by centrifugation and frozen at -20 °C.

### Protein Complex Purification

Cell pellets were suspended in equilibration/wash buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 15 mM imidazole, pH 7.8), sonicated, and passed through a French press to lyse the cells. Nucleic acids were removed by the addition of DNase. The cell lysate was centrifuged at 34540 g for 1 h at 4 °C. The supernatant was filtered with a 0.45 µm filter from Millipore and loaded onto a nickel affinity column pre-equilibrated with equilibration/wash buffer and then washed with ten column volumes of the same buffer. The protein complex was eluted using a segmented gradient starting with eight column volumes of 0-10% elution buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 250 mM imidazole, pH 7.8), followed by 12 column volumes of 10-100% elution buffer



and finally eight column volumes of 100% elution buffer. Protein complex from the nickel affinity column was then concentrated via Amicon Centrifugal Filters at 3889 g and loaded onto a Hi-Load 16/60 Superdex 200 column pre-equilibrated with 10 mM Hepes, 500 mM KCl, pH 7.8 for purification on a TSK gel G3000SW<sub>XL</sub> column pre-equilibrated with 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 10 mM Hepes, pH 6.8 for analysis. Fractions containing pure BCCP-BC complex were concentrated and stored at -80 °C.

### Crystallization

Crystals were obtained by sitting drop vapor diffusion at room temperature using a 10 mg/mL solution of the BCCP-BC complex. The crystallization condition contained a reservoir solution of 0.2 M ammonium sulfate, 0.1 M BIS-TRIS (pH 6.5), and 25% w/v PEG 3350. The sitting drop contained a well to protein ratio of 1.75:2  $\mu$ L. Crystals appeared after three months and were cryoprotected with a 30% v/v ethylene glycol/reservoir solution and then mixing that solution with crystal mother liquor in a 1:1 ratio. The crystal was submerged in the cryoprotectant and then immediately flash-cooled in liquid nitrogen.

### X-ray Data Collection, Structure Determination, and Refinement

X-ray diffraction data were collected to 2.49 Å at the Advanced Photon Source beamline NE-CAT 24-ID-E equipped with the ADSC Q315r detector. The data was processed and scaled using XDS (Kabsch, 2010). The crystal belonged to the C2 space group and had unit cell parameters  $a = 233.0$  Å,  $b = 96.4$  Å,  $c = 120.6$  Å and  $\beta = 120.2^\circ$ . The asymmetric unit contained two BC homodimers and each monomer of BC was in complex with one BCCP molecule. The phase information was obtained by molecular replacement using Phaser (Adams *et al.*, 2010) and a BCCP-BC heterodimer search model that was acquired from the solution of a 3.7 Å data set of

a different crystal form (Table 3.1). The molecular replacement solution was further refined using several cycles of phenix.refine (Adams *et al.*, 2010) and manual rebuilding in COOT (Emsley and Cowtan, 2004). All water molecules and ions added were manually verified after the final refinement. The final model consists of residues 1-163 and 167-446 for BC molecule A, 77-156 for BCCP molecule B, 1-161, 170-182, and 196-446 for BC molecule C, 79-156 for BCCP molecule D, 1-159,170-183, and 197-446 for BC molecule E, 1-160, 169-184, and 196-446 for BC molecule F, 80-156 for BCCP molecule G, 80-156 for BCCP molecule I, and 455 water molecules. Twelve sulfate ions and one ethylene glycol molecule were included in the final model based on the shape of the difference electron density, composition of the crystallization and cryoprotectant solutions, and the refined B-factors. Data collection statistics are shown in Table 3.1.

#### Crystal Structure Determination (P2<sub>1</sub> Crystal Form)

The molecular replacement procedure was applied to locate a solution. First, a monomer of biotin carboxylase (PDB accession code 1DV1) was used as a search model. Four monomers were located by using the program MOLREP (Bailey, 1994). Their positions were fixed and a monomer of the biotin carboxyl carrier protein (PDB accession code 1BDO) was used in the additional molecular replacement search with MOLREP. Four BCCP monomers were located in the asymmetric unit producing a hetero-octamer for the BCCP-BC complex. The positioned MR model was refined in REFMAC (Bailey, 1994) using local non-crystallographic restraints and “jelly” body refinement which resulted in  $R = 20.70$  and  $R_{\text{free}} = 31.15\%$ . No model building or additional refinement has been performed because of low resolution of the data set (3.74 Å) and our further structure determination attempts were directed into the improvement of the resolution limits of the crystals.

Table 3.1 Data Collection and Refinement Statistics for BCCP-BC Structures

<b>Data Collection</b>		
Space group	C2	P2 <sub>1</sub>
Cell dimensions		
a, b, c (Å)	233.0, 96.4, 120.6	100.6, 200.7, 121.3
β (°)	120.2	104.1
Resolution (Å)	2.49	3.74
R <sub>sym</sub> <sup>ab</sup>	6.2 (33.1)	19.2 (65.9)
I / σI	11.2 (2.4)	7.2 (2.6)
Completeness (%)	98.5 (93.7)	98.3 (92.4)
Redundancy	2.5 (2.5)	4.3 (4.0)
<b>Refinement</b>		
Resolution (Å)	104.3-2.49	
Number of reflections	79,181	
R <sub>work</sub> / R <sub>free</sub> <sup>c</sup>	19.50/22.91	
Number of atoms		
Protein	15,643	
Ligand/ion	64	
Water	455	
B-factors		
Protein	39	
Ligand/ion	34	
Water	26	
RMSD		
Bond lengths (Å)	0.003	
Bond angles (°)	0.72	

<sup>a</sup>Values in parentheses are for the highest-resolution shell.

<sup>b</sup>R<sub>sym</sub> = (Σ|I<sub>i</sub> - <I<sub>i</sub>>| / Σ|I<sub>i</sub>|) \* 100, where I<sub>i</sub> is the intensity of the i<sup>th</sup> observation and <I<sub>i</sub>> is the mean intensity of the reflection.

<sup>c</sup>R = (Σ||f<sub>o</sub>| - |f<sub>c</sub>|| / Σ|f<sub>o</sub>|) \* 100, where F<sub>o</sub> and F<sub>c</sub> are the observed and calculated structure factor amplitudes, respectively. R<sub>free</sub> is calculated using 5% of reflections omitted from the refinement.

## Programs

The programs used for calculating RMSDs and accessible surface areas were PDBefold (Krissinel and Henrick, 2004) and PISA (Krissinel and Henrick, 2007), respectively.

## Accession Number

The atomic coordinates and structure factors for the BCCP-BC crystal structure have been deposited in the Protein Data Bank under accession number 4HR7.

## Results and Discussion

### Co-purification of BCCP and BC

The genes encoding BCCP (*accB*) and BC (*accC*) are located in an operon in the *E. coli* chromosome and are co-transcribed (James and Cronan, 2004). To mimic the *in vivo* arrangement, *accB* and *accC* were coexpressed using a minioperon where a histidine-tag was put on the N-terminus of BCCP. After purification by nickel affinity and size exclusion chromatography, the major peak contained the BCCP-BC complex, which was used for crystallization (Figure 3.1 A). Gel electrophoresis showed a band at 50 kDa corresponding to the BC monomer and a band at ~22 kDa that is indicative of full length BCCP (Figure 3.1 B). The BCCP runs slightly higher than its 16.7 kDa molecular weight; this is thought to be caused by a pro/ala rich linker in the middle of the protein (Li and Cronan, 1992). Most importantly, the fact that the BCCP-BC complex remains intact after both nickel affinity and size exclusion chromatography suggests the BCCP-BC interaction is indeed stable.

### Quaternary Structure

The overall structure of the BCCP-BC complex has  $D_2$  symmetry and is shown in Figure 3.2 A-C. One of the most striking features of the BCCP-BC complex is that two BC homodimers (which have  $C_2$  symmetry) come together to form a tetramer. If the BC tetramer is viewed by looking at one homodimer on top of the other it becomes readily apparent that the homodimers are rotated approximately  $90^\circ$  with respect to one another (Figure 3.2 C). Tetrameric BC, in the BCCP-BC complex is in stark contrast to the structures reported to date of BC, which have shown the enzyme to be a homodimer with  $C_2$  symmetry (Chou *et al.*, 2009; Mochalkin *et al.*, 2008; Thoden *et al.*, 2000; Waldrop *et al.*, 1994). Moreover, there are no packing interfaces in

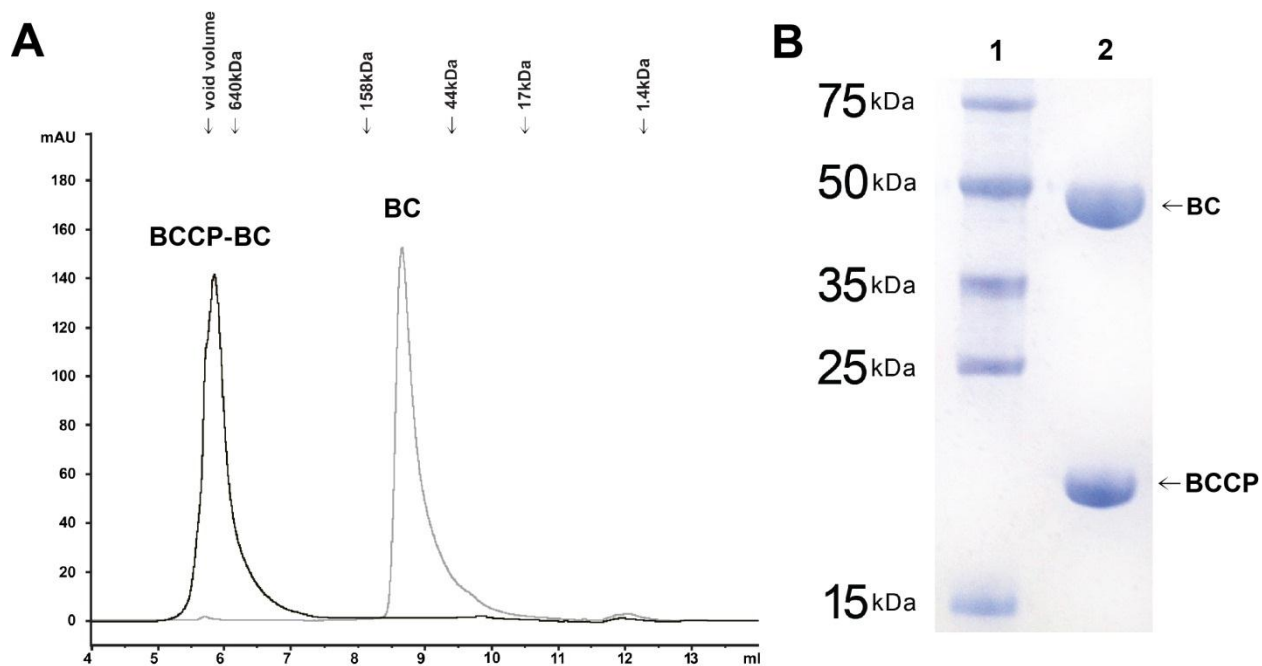


Figure 3.1 Purification and SDS-PAGE analysis of the BCCP-BC complex. **A)** Size exclusion chromatography profile showing mAU vs. elution volume of proteins chromatographed on a Tosoh TSK gel G3000SW<sub>XL</sub> column. Arrows at the top of the graph indicate peak elution volumes of molecular weight standards and blue dextran. BCCP-BC complex and BC profiles are shown as black and grey lines, respectively, and are depicted by name above their elution peak. **B)** SDS-PAGE gel showing molecular weight standards in lane 1 and lane 2 exhibiting full length BCCP (22kDa) and BC (50kDa) from the BCCP-BC gel filtration peak in **A**. This figure represents the protein used to crystallize the structure shown in Figure 3.2.

the crystals of BC alone that display the same dimer-dimer interaction as the BCCP-BC complex.

The tetramer of BC is bound to four BCCP molecules where each BCCP molecule forms two interfaces. One interface is with a BC monomer located in the upper homodimer while the other is with a BC monomer in the lower homodimer of the tetramer (Figure 3.2 A and B). Thus, the four BCCP molecules essentially act as molecular clips to hold two BC homodimers together to form a tetramer. It is important to note that the stoichiometry of two BC dimers plus four BCCP molecules observed here does not agree with the stoichiometry previously reported of one

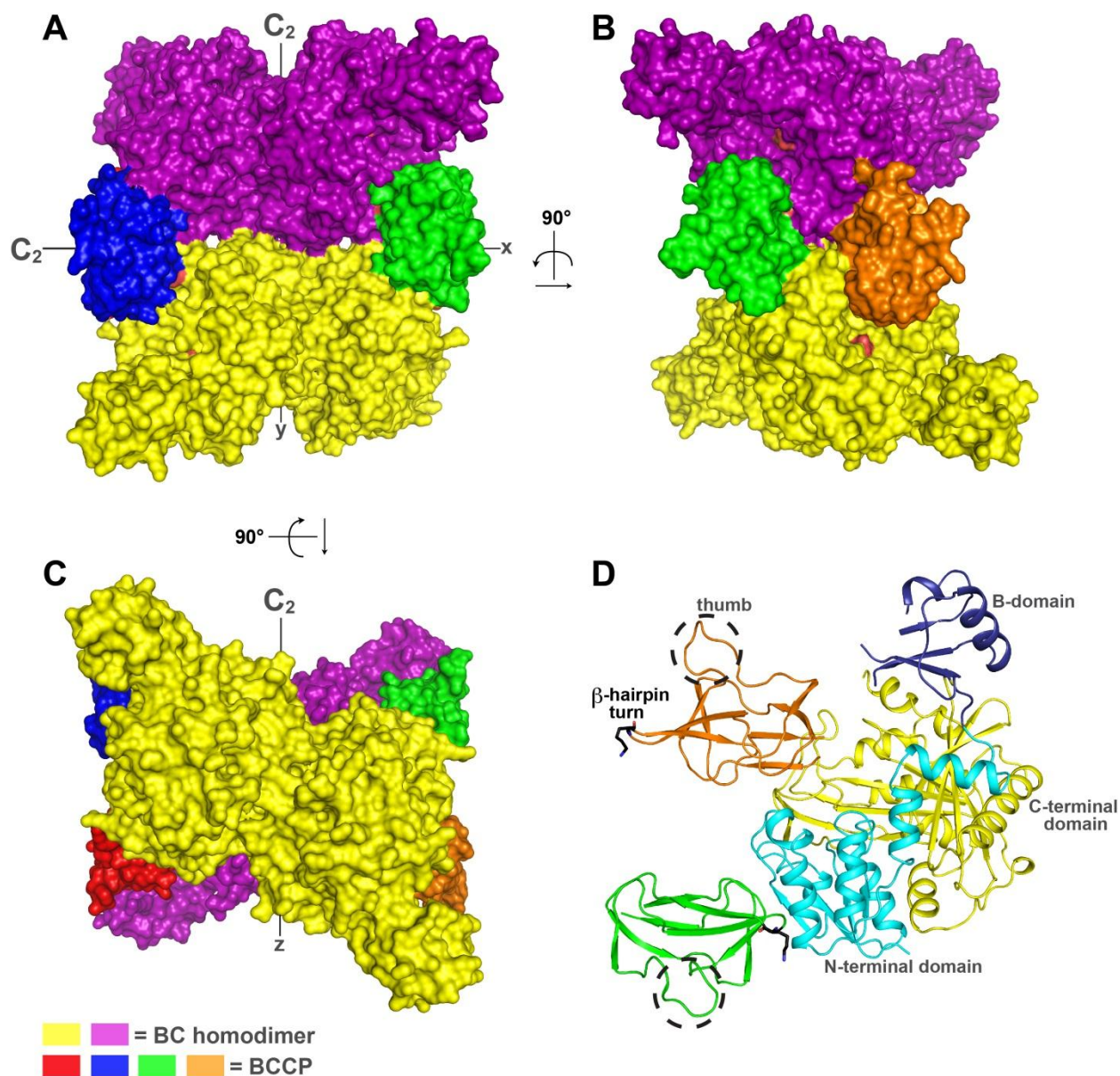


Figure 3.2 Quaternary structure of the BCCP-BC complex. **A, B and C)** Surface rendering of the BCCP-BC complex showing the BC homodimer in purple (top) and yellow (bottom) with individual BCCPs shown in blue, green, orange, and red. **B** is **A** rotated 90 ° to the left and **C** is **A** rotated 90 ° around the x-axis in a clockwise motion. The local symmetry axes of the BCCP-BC complex are also indicated in **A** and **C**. **D)** Cartoon representation of a monomer of BC with the two interfacial BCCP molecules in orange and green, respectively. The N-terminal domain, B-domain, and the C-terminal domain of the BC monomer are labeled and colored in light blue, dark blue, and yellow, respectively. Lys122 is represented as black sticks in each BCCP molecule with oxygen and nitrogen atoms in red and blue, respectively. The thumb of each BCCP molecule is designated by dashed circles.

BC dimer and four BCCP molecules (Choi-Rhee and Cronan, 2003). Moreover, these investigators also concluded that the N-terminal domain of BCCP was required for the interaction of BCCP with BC (Choi-Rhee and Cronan, 2003). As can be seen in Figure 3.2 D it is the C-terminal region of BCCP that interacts with BC which is consistent with kinetic studies showing the C-terminal domain is a substrate for BC (Blanchard *et al.*, 1999a). Electron density for the N-terminal region of BCCP was not observed presumably due to inherent flexibility in this region of the molecule. Control experiments in which crystals of the BCCP-BC complex were run on SDS-PAGE show that the N-terminal domain is, in fact, attached to the C-terminal domain. The high degree of flexibility of the N-terminal domain of BCCP was also observed (by the lack of NOEs) in the N-terminal region of the 1.3S subunit of the homologous biotin-dependent enzyme transcarboxylase (Reddy *et al.*, 1998). The N-terminal region of the 1.3S subunit is involved in contacts with the other subunits of transcarboxylase (Carey *et al.*, 2004) and by inference the N-terminal domain of *E. coli* BCCP may also interact with the CT subunit of acetyl-CoA carboxylase. Moreover, the fact that the N-terminal domain of BCCP is not involved in binding to BC is consistent with the observations that this domain down regulates transcription of the *accBC* operon (James and Cronan, 2004). Thus, if BCCP and BC form a complex *in vivo*, then it is reasonable to assume that the N-terminal domain of BCCP must remain unhindered so it could bind DNA and act as a transcriptional repressor.

The quaternary structure shown here of two BC homodimers and four BCCP molecules has a molecular weight of ~260 kDa. Yet, the size exclusion chromatography data show a molecular weight greater than 640 kDa for the BCCP-BC complex (Figure 3.1 A). Thus, the structure observed here is likely a monomer of a much larger complex. For instance, if the ~260 kDa BCCP-BC complex shown here were a trimer it would have a molecular weight of ~800

kDa, which would be consistent with the three-fold axis of symmetry observed in two other biotin-dependent enzymes, propionyl-CoA carboxylase (Huang *et al.*, 2010) and methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012).

Several lines of evidence indicate that the BCCP-BC complex described here is physiologically relevant. First, the minioperon of BCCP-BC used in this study mirrors the operon and cotranscription of these two proteins that normally occurs in the cell. The reason that only a homodimer of biotin carboxylase has been crystallized before is because the gene for the enzyme was overexpressed in the absence of BCCP which does not mimic the *in vivo* situation. Moreover, the homodimer of biotin carboxylase observed in previous crystal structures is part of the homotetramer observed here where the interaction of two homodimers forms a tetramer that is mediated by BCCP. Second, the formation of a BCCP-BC complex is also consistent with the observations that BCCP by itself forms aggregates (Choi-Rhee and Cronan, 2003; Nenortas and Beckett, 1996). Thus, formation of a complex between BCCP and BC is more likely to be the case *in vivo* rather than BC existing as a homodimer and BCCP as an aggregate. Third, the BCCP-BC complex presented here is unlikely to be a consequence of the crystallization conditions because the same structure was observed under different crystallization conditions and in a different crystal form albeit at a lower resolution (3.7 Å) (Table 3.1).

### BCCP Structure

The overall structure of the C-terminal domain of BCCP bound to BC is virtually identical to the crystal (Athappilly and Hendrickson, 1995) and NMR (Yao *et al.*, 1997) structures of the C-terminal domain of BCCP. The root-mean-square deviations (RMSD) between the equivalent C $\alpha$  atoms of BCCP molecule B in the BCCP-BC complex and the crystal



and NMR structures were 0.64 Å and 1.28 Å, respectively. When the *E. coli* BCCP molecules observed in the complex were superimposed to the C $\alpha$  atoms of the BCCP domain from human ACC2 (Lee *et al.*, 2008) and the 1.3S subunit of transcarboxylase from *Propionibacterium shermanii* (Reddy *et al.*, 1998), the RMSD was 1.7 Å and 1.5 Å, respectively. Although, the RMSDs were calculated between the C $\alpha$  atoms of matching residues, the major difference between these molecules is the lack of a “thumb” in the non-*E. coli* proteins (Figure 3.3). This “motif” is not involved in interacting with BC and extends out away from the main body of the BCCP-BC complex (Figure 3.2 D). Mutations in this region of *E. coli* BCCP were found to impair the growth of *E. coli* suggesting that it is important for the function of the protein; however, the chemical mechanism for this phenotype is not understood (Cronan, 2001).

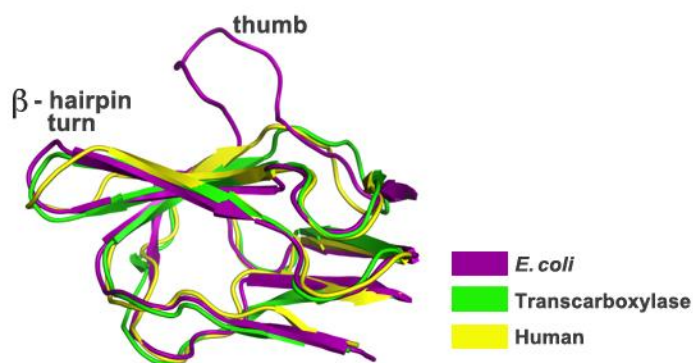


Figure 3.3 Superposition of BCCP. Superposition of *E. coli* BCCP from the BCCP-BC complex (purple) with the BCCP domain from human acetyl-CoA carboxylase (yellow) and the 1.3S subunit of transcarboxylase (green). The  $\beta$ -hairpin turns can be seen pointing to the left and the thumb of the *E. coli* BCCP is “thumbs up” toward the top of the figure.

The hairpin turn in BCCP that contains the lysine residue that is biotinylated also protrudes away from the main body of the BCCP-BC complex (Figure 3.2 D). The C $\alpha$  of Lys122

is about  $\sim 40$  Å from Arg338, which is an active site residue that is important for catalysis (Sloane and Waldrop, 2004). The C $\alpha$  of Lys122 was used as a reference because no electron density for the side chain of this residue was observed. This distance is consistent with the structures of related biotin-dependent holoenzymes, propionyl-CoA carboxylase, pyruvate carboxylase and methylcrotonyl-CoA carboxylase, which have distances between the BC and CT active sites of 55, 75 and 80 Å, respectively (Huang *et al.*, 2012; Huang *et al.*, 2010; Xiang and Tong, 2008). Considerable movement of the BCCP domain has been proposed for catalysis by other biotin-dependent carboxylases (Huang *et al.*, 2012; St Maurice *et al.*, 2007; Xiang and Tong, 2008) and BCCP has been observed in different positions in the cryo-electron microscopy studies on propionyl-CoA carboxylase (Huang *et al.*, 2010). Thus, it is not surprising that the *E. coli* BCCP is  $\sim 40$  Å away from the BC active site and may possibly be equidistant from where the BC and CT active sites would be in a full structure ( $\sim 80$  Å).

The large distance between biotinylated BCCP and the active site is also in accord with reports that the BCCP-BC complex exhibits little to no catalytic activity in the absence of CT (Alves *et al.*, 2011; Soriano *et al.*, 2006). Measurements of the enzymatic activity of the BCCP-BC complex used in this study confirm the previous studies in that the activity of the BCCP-BC complex was 0.005  $\mu\text{mol}/\text{min}/\text{mg}$ , while the activity in the presence of CT and acetyl-CoA increased markedly (0.530  $\mu\text{mol}/\text{min}/\text{mg}$ ) suggesting that BCCP in the complex was at least partially biotinylated. Thus, the BCCP-BC complex is catalytically competent but only when complexed with CT and acetyl-CoA. The fact that the BCCP-BC complex is only active in the presence of CT and acetyl-CoA is important for the regulation of enzyme activity because it means that ATP will only be hydrolyzed when acetyl-CoA is present. This is a classic example of substrate synergism in which the wasteful hydrolysis of ATP is prevented unless all the

substrates are present. The structure reported here provides a structural context for understanding why the BCCP-BC complex is inactive in that the loop containing Lys122 to which biotin is attached is far away from the active site of biotin carboxylase. How the activity of the BCCP-BC complex is increased in the presence of CT and acetyl-CoA will have to await the structure of holo-acetyl-CoA carboxylase.

The fact that the hairpin loop is exposed to the solvent is also consistent with the observation that it is not biotinylated. Lys122 in BCCP is biotinylated by biotin protein ligase and, therefore, must be readily accessible *in vivo*. A superposition of the structure of *Pyrococcus horikoshii* biotin protein ligase complexed with BCCP (Bagautdinov *et al.*, 2008) with the *E. coli* BCCP in the BCCP-BC complex shows the biotin protein ligase rests alongside BC with very little steric overlap (Figure 3.4). Thus, the structure of the BCCP-BC complex observed here is exactly what would be expected if the BCCP is not biotinylated.

### BC Structure

The structure of the BC component in the BCCP-BC complex is virtually identical to previously determined structures of the BC homodimer (Chou *et al.*, 2009; Mochalkin *et al.*, 2008; Thoden *et al.*, 2000; Waldrop *et al.*, 1994). A superposition of the BC homodimer with one of the BC homodimers in the complex yielded a RMSD of 0.76 Å. BC is a member of the ATP-grasp superfamily of enzymes that all have a similar tertiary structure composed of three domains: N-terminal, C-terminal, and B-domain (Figure 3.2 D) (Galperin and Koonin, 1997). The N- and C-terminal domains form the main body of the BC monomer while the B-domain either extends away from the other domains in the absence of ATP or rotates 45° to cover the

active site in the presence of ATP (Thoden *et al.*, 2000). In the BCCP-BC complex the B-domain is splayed open and therefore consistent with no substrates being bound.

Although the BCCP-BC complex described here did not contain any substrates, there was electron density in each BC active site that could be represented as an anion the size of phosphate or sulfate. Phosphate anions have been observed in structures of homodimeric BC (Thoden *et al.*, 2000; Waldrop *et al.*, 1994); however, sulfate ions were modeled in the BCCP-BC complex due to the high concentration of ammonium sulfate in the crystallization solution.

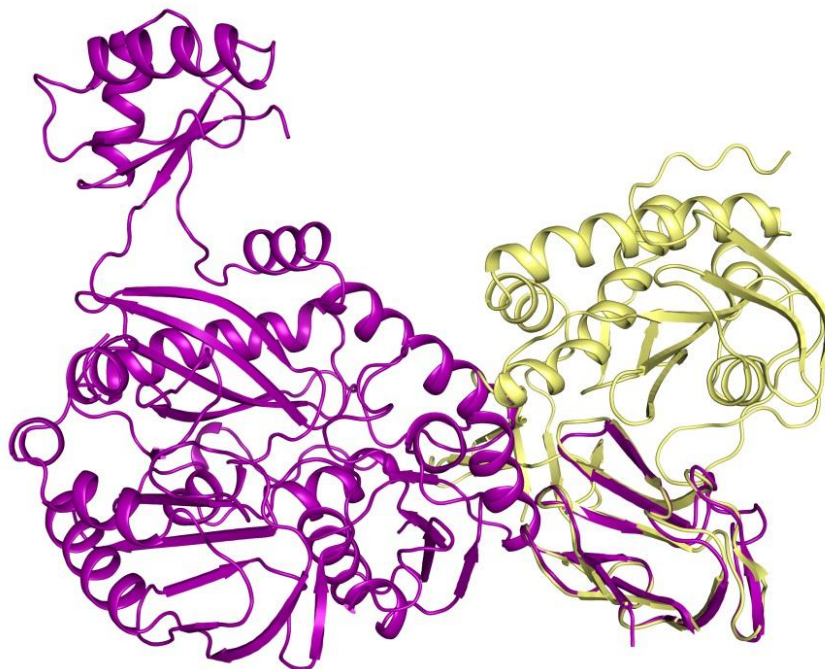


Figure 3.4 Superposition of BCCP from the *E. coli* BCCP-BC complex and the BCCP-biotin protein ligase complex from *P. horikoshii*. Biotin carboxyl carrier protein-biotin carboxylase complex is shown in purple and the biotin carboxyl carrier protein-biotin protein ligase complex is shown in gold. The superposition of the unbiotinylated BCCPs depicts a conformational state of the BCCP-BC complex by which biotin protein ligase can biotinylate the BCCP-BC complex.

## BCCP-BC Interfaces

BCCP binds to BC via two different interfaces. In the first interface, BCCP binds to the N-terminal domain of BC. The amino acids involved are located at the C-terminus of strands  $\beta 2$  and  $\beta 3$  in BC and a loop connecting two  $\beta$  strands in BCCP (Figure 3.5 A and B). One interaction involves the carbonyl oxygen of Cys50 from BC forming a hydrogen bond with the peptidic NH of Phe148 of BCCP, while in a second interaction the peptidic NH of Cys50 hydrogen bonds to the carboxylate group of Glu147 from BCCP. In addition, the side chains of His32 and Thr48 from BC interact with the carboxylate group of Glu147 from BCCP via a water molecule. Consistent with Glu147 being involved in binding to BC is that mutation of Glu147 to Lys (Chapman-Smith *et al.*, 1999) in the domain comprising the C-terminal 87 residues of BCCP, which does exhibit activity with BC, (Blanchard *et al.*, 1999a) resulted in over a 100-fold increase in the  $K_m$  (unpublished data).

In the second interface the opposite end of the BCCP molecule interacts with residues in the N- and C-terminal domains of BC located in the opposite half of the BC tetramer (Figure 3.5 A and C). Residues involved in this interface stem from the  $\beta 1$  strand of BCCP and the BC residues 56 and 57 located in a loop along with amino acids in the turn between two  $\beta$  strands. Specifically, the carboxylate of Asp149 of BCCP forms a hydrogen bond with the hydroxyl group of Tyr380 from BC (Figure 3.5 C). The rest of the interactions at this interface are mediated through a putative sulfate ion and a water molecule (Figure 3.5 C). The presumed sulfate ion likely replaced a phosphate group, which was the case observed in the active site of BC described above. The residues in BC involved in this interaction are the side chain of Ser56 and the peptidic NH group of Tyr381, while in BCCP the interacting groups were the side chain of Arg84 and the peptidic NH group of Ile82. It is important to note, the BCCP-BC complex was

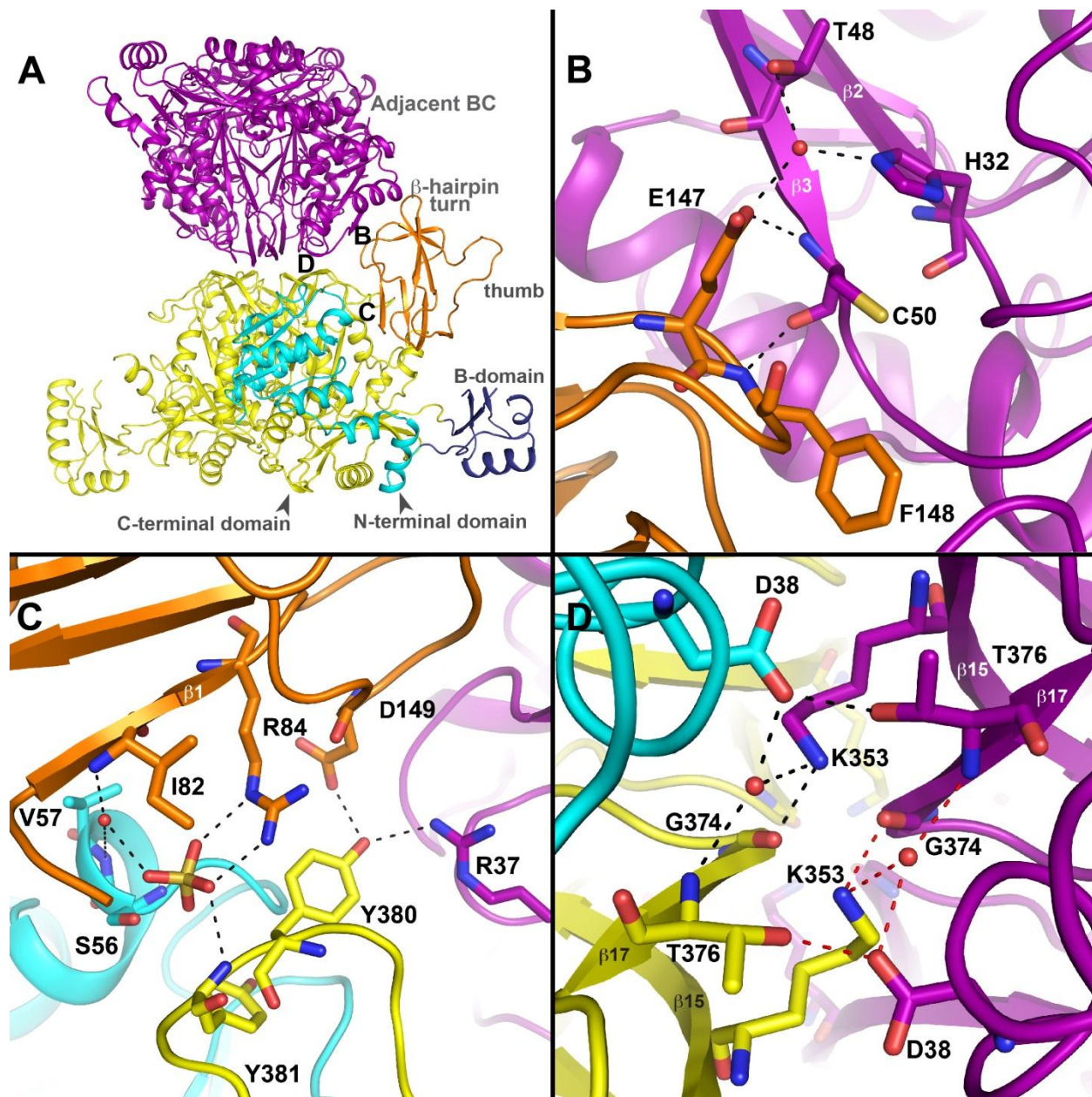


Figure 3.5 The BCCP-BC complex interfaces. Oxygen, nitrogen, sulfur atoms, and water are depicted in red, blue, gold, and red spheres, respectively. Residues are indicated by their single letter abbreviation followed by their residue number in *E. coli*. Dashed lines depict hydrogen bonds and fall within 2.41-3.25 Å. The color scheme remains constant throughout the figure. **A**) BC homodimers are shown in purple (top) and yellow (bottom) of the complex. The N-terminal domain, B-domain and C-terminal domain of one monomer of the yellow homodimer are labeled and colored light blue, dark blue, and yellow, respectively. One of the BCCP's binding these two homodimers together is shown in orange with the  $\beta$ -hairpin turn pointing to the purple homodimer. **B**, **C**, and **D** correspond to the area that is enlarged in **B**, **C**, and **D**, respectively. **B** and **C**) A magnified view of the two BCCP-BC interfaces. **D**) A magnified view of the BC-BC interface.

stable to gel filtration chromatography in a buffer not containing sulfate and with a molecular weight consistent with a multimeric quaternary structure (Figure 3.1 A).

The interactions observed in the *E. coli* BCCP-BC complex are similar to those observed for other biotin-dependent carboxylases in which structural information about the interaction of BCCP with either BC or CT is available (Fan *et al.*, 2012; Huang *et al.*, 2012; Huang *et al.*, 2010; Lietzan *et al.*, 2011; St Maurice *et al.*, 2007; Xiang and Tong, 2008). The interfaces between BCCP and BC or CT in other biotin-dependent carboxylases were small and composed mostly of hydrogen and ionic bonds. Moreover, the residues involved in the protein-protein interactions were not conserved. The explanation given for the relatively weak BCCP-BC interface interactions was that BCCP has to undergo a large translocation between the BC and CT active sites. If the interface interactions were strong, indicative of hydrophobic interactions, this would create a thermodynamic well preventing or slowing the ability of BCCP to translocate to the CT binding site. In fact, proteins that associate in a transient or non-obligatory manner show a general trend of interaction through hydrogen and ionic bonds instead of hydrophobic contacts (Nooren and Thornton, 2003; Ozbabacan *et al.*, 2011).

The similarities of the BCCP-BC interfaces between the *E. coli* proteins and other biotin-dependent carboxylases are striking. The individual interfaces in the *E. coli* BCCP-BC complex are small, with buried surface areas of 240 Å<sup>2</sup> for the interface with the putative sulfate ion and 480 Å<sup>2</sup> for the other. However, these two small interfaces are repeated four times in the complex giving a total buried surface area of 2880 Å<sup>2</sup>. Both interfaces are composed of hydrogen bonds and ionic interactions with no hydrophobic contacts. Moreover, the interface residues are not strictly conserved across a spectrum of Gram positive and Gram negative bacteria with the only exceptions being Cys50 and Tyr381 in BC and Glu147 in BCCP (Figure 3.6).



While the interfaces in the *E. coli* BCCP-BC complex are apparently strong enough to keep the complex intact on a gel filtration column, the small buried surface area for an individual BCCP molecule and the lack of hydrophobic interactions likely provide the appropriate amount of flexibility for movement of BCCP between the BC and CT active sites. Exactly how BCCP moves between the BC and CT active sites is not known. It might involve translocation of the entire BCCP as proposed in pyruvate carboxylase (St Maurice *et al.*, 2007; Xiang and Tong, 2008), propionyl-CoA carboxylase (Huang *et al.*, 2010), and methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012) which is consistent with the lack of hydrophobic residues because any partial dissociation of BCCP would not expose any hydrophobic residues to solvent.

	Biotin Carboxylase										Biotin Carboxyl Carrier Protein					
	32	34	37	48	50	56	353	374	376	380	381	84	96	147	148	149
<i>Escherichia coli</i>	H	S	R	T	C	S	K	G	T	Y	Y	R	S	E	F	D
<i>Haemophilus influenzae</i>	H	T	R	T	C	S	K	G	T	H	Y	R	S	E	F	D
<i>Pseudomonas aeruginosa</i>	H	T	R	S	C	A	K	G	S	N	Y	R	S	E	F	D
<i>Moraxella catarrhalis</i>	Y	T	K	A	C	S	Q	G	T	Y	Y	T	S	E	F	G
<i>Neisseria gonorrhoeae</i>	H	E	K	S	C	S	P	G	R	Y	Y	K	G	E	F	G
<i>Enterococcus faecalis</i>	Y	E	Q	A	C	A	K	G	S	Y	Y	T	A	E	F	G
<i>Streptococcus pneumoniae</i>	Y	T	K	A	C	A	K	G	T	Y	Y	E	G	E	F	G
<i>Staphylococcus aureus</i>	Y	K	E	A	C	T	K	G	Q	Y	Y	K	S	E	Y	D

Figure 3.6 Interface residues in *E. coli* BC and BCCP compared to homologous residues in other species. The number above the column denotes the residue number in *E. coli* BC or BCCP. Letters in red indicate a strictly conserved residue with respect to that found in *E. coli* BC or BCCP. The organism names are highlighted in pink and purple representing a negative or positive Gram reaction, respectively. This figure relates residues shown in Figure 3.5 to homologous residues in pathogenic bacteria.

### BC-BC Interface

The BCCP-BC complex is also stabilized by a BC-BC interface. The buried surface area for the interface is small,  $350 \text{ \AA}^2$ , suggesting a weak interaction; however, there are two identical BC-BC interfaces, which effectively doubles the interaction energy (Figure 3.5 A and D). In fact, if the total buried surface area for the two BC-BC interfaces ( $700 \text{ \AA}^2$ ) is combined with the total



buried surface area for the BCCP-BC interfaces the total buried surface area for the complex is 3580 Å<sup>2</sup> which is consistent with the BCCP-BC complex being a permanent or obligate protein-protein interaction (Ozbabacan *et al.*, 2011). While the dissociation constant between BC and BCCP has not been determined this level of total buried surface area suggests it would be <10<sup>-6</sup> M (Ozbabacan *et al.*, 2011).

The interacting residues of the BC monomer are located in the C-domain in β-strands 15 and 17 with the exception of Asp38, which is in the N-terminal domain of BC. The residues that form the interface are duplicated such that there is two-fold symmetrical relationship for the interactions at each interface (Figure 3.5 D). The two most prominent interactions are between the carbonyl oxygen of the strictly conserved Gly374 (Figure 3.6) and the side chain of Lys353 and a hydrogen bond between the side chains of Thr376 and Asp38, which are shown in Figure 3.5 D.

#### Comparison to BCCP-BC Structures from Other Biotin-Dependent Enzymes

While the structure presented here is the leading model of the BCCP-BC complex for acetyl-CoA carboxylase, the structure of BCCP and BC has been determined for related biotin-dependent carboxylases such as pyruvate carboxylase (Lietzan *et al.*, 2011; St Maurice *et al.*, 2007; Yu *et al.*, 2009), propionyl-CoA carboxylase (Huang *et al.*, 2010), methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012), and urea carboxylase (Fan *et al.*, 2012). What distinguishes these enzymes from bacterial acetyl-CoA carboxylase is that the BC, BCCP and CT functionalities are either all part of a single polypeptide chain or the BCCP and BC domains are located on the same polypeptide.

The only holoenzyme structure that shows an interaction between the BC and BCCP domains is the structure of pyruvate carboxylase from *Rhizobium etli* (Lietzan *et al.*, 2011; St Maurice *et al.*, 2007). The interaction in the C-domain between BC and BCCP in *R. etli* pyruvate carboxylase is not the same C-domain interaction observed in the *E. coli* BCCP-BC complex (Figure 3.7 A). The reason for this difference becomes obvious in Figure 3.7 B which shows that in pyruvate carboxylase from *R. etli* there is a loop comprising residues Val52-Gly63 that protrudes from the main body of the protein at exactly the spot that the bacterial BCCP would bind to BC. Thus, the *E. coli* BCCP-BC complex described above does not have any similarities to BCCP-BC interactions observed in other biotin-dependent carboxylases. The apparent uniqueness of the bacterial BCCP-BC interaction is likely to be a consequence of not being

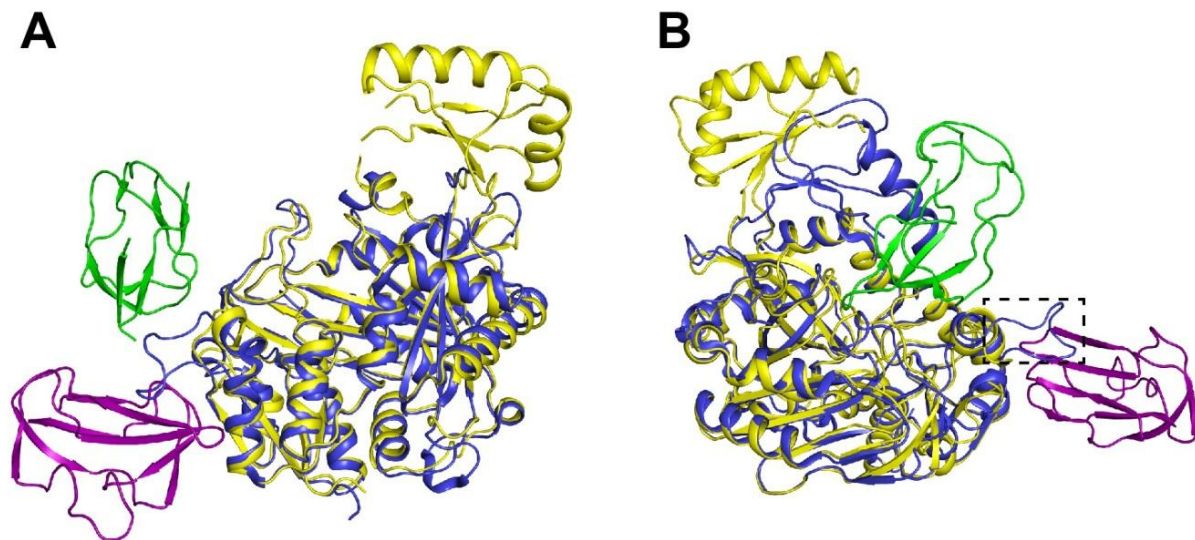


Figure 3.7 Biotin carboxylase from *E. coli* superimposed onto the biotin carboxylase domain of *R. etli* pyruvate carboxylase. The BCCP-BC complex is shown in purple and gold, respectively, and the BCCP and BC domains of *R. etli* pyruvate carboxylase are shown in blue and green. **A)** Overlay of BC from *E. coli* with the BC domain from *R. etli* pyruvate carboxylase, PDB ID = 2QF7. **B)** Overlay of BC from *E. coli* with the BC domain from *R. etli* pyruvate carboxylase, PDB ID = 3TW6. The dashed box indicates the loop in *R. etli* pyruvate carboxylase that would hinder *E. coli* BCCP binding.

covalently linked like the structures of similar biotin-dependent enzymes and, therefore, BCCP must be tethered to BC through non-covalent interactions. The differences observed here, however, do not preclude the possibility that the way BCCP interacts with the active site of BC is similar to pyruvate carboxylase (Lietzan *et al.*, 2011).

### Implications for Antibacterial Development

The dramatic increase in the number of pathogenic bacteria with extensive resistance to antibiotics has been well documented (Nathan, 2012; Spizek *et al.*, 2010). Thus there is a pressing need for new targets and biotin carboxylase has been validated as a novel target for antibacterial development (Miller *et al.*, 2009; Mochalkin *et al.*, 2009). Given that the protein-protein interface in *E. coli* BCCP-BC appears to be unique and presumably required for enzymatic activity, this greatly expands the number of potential target sites beyond just the traditional active sites.

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## CHAPTER 4 CONCLUSIONS

### Summary

This dissertation addressed whether the subunits of *E. coli* acetyl-CoA carboxylase undergo the two half-reactions catalyzed by the enzyme separately or as a multifunctional complex. Specifically, does the BCCP interact with BC and CT separately to undergo the two half-reactions required to form malonyl-CoA, or do all three subunits come together to form a macromolecular complex? Since the late 1960's, it has been assumed that a complex is formed but there has been minimal biochemical evidence to support this claim (Alberts *et al.*, 1969; Fall and Vagelos, 1972; Nenortas and Beckett, 1996).

Several techniques were utilized to show that acetyl-CoA carboxylase forms a complex. The array of enzyme kinetic studies showed that binding and catalysis at the BC active sites affects binding and catalysis at the CT active sites and vice versa. The BCCP-BC complex, which is likely the *in vivo* form of these proteins because free BCCP forms aggregates, was shown to not hydrolyze ATP unless CT and acetyl-CoA were present. To determine if the complex formed and if the presence or absence of ATP and/or acetyl-CoA had an effect on complex formation, co-immunoprecipitation assays were performed. The results confirmed that these components form a complex *in vitro* and the complex formation is not dependent on acetyl-CoA or ATP analogs. Furthermore, *in vivo* overexpression of all four genes encoding the subunits of ACC form a functional ACC complex. These results suggested that all three components come together to form a complex that does not hydrolyze ATP until acetyl-CoA induces ACC to increase its catalytic reaction.



The next step was to determine the three-dimensional structure of all or half of the acetyl-CoA carboxylase complex. To this end, the three-dimensional structure of the BCCP-BC complex was determined by X-ray crystallography. This is the first complex of two ACC subunits observed to date. This structure showed three new interfaces formed by the two proteins. Four BCCP molecules each form two interfaces with different BC monomers, effectively clipping the complex together. The third interface is formed between the two BC homodimers that are clipped together. The unbiotinylated BCCP molecules were shown to be in a position where the BCCP-BC complex could interact with biotin protein ligase in order to attach the prosthetic group. Additionally, the large distance of the  $\beta$ -hairpin turn of BCCP to the active site provides structural evidence as to why BCCP-BC does not hydrolyze ATP in the absence of CT and acetyl-CoA. The BCCP and BC subunits superimpose well to previously determined biotin-dependent carboxylase structures but small differences like the loop extending out of the BC domain of *R. etli* pyruvate carboxylase would prevent the BCCP interaction with BC observed in the BCCP-BC complex. This complex is enzymatically active in the presence of CT and acetyl-CoA, which supports that the BCCP-BC structure is all or part of the functionally relevant molecule in *E. coli*.

These studies provide additional information on the recently proposed mechanism of CT regulation by Meades *et al.* (2010). Since both the BCCP-BC and ACC complexes form *in vivo* but do not hydrolyze ATP in the absence of acetyl-CoA, this presents a new regulatory archetype for acetyl-CoA carboxylase (Figure 4.1). During stationary phase when acetyl-CoA levels are low, the ACC complex does not hydrolyze ATP and binds the mRNA of its  $\alpha$ -CT and  $\beta$ -CT subunits to attenuate translation. During log phase when acetyl-CoA levels are high, acetyl-CoA outcompetes mRNA for the CT active site to initiate fatty acid and CT synthesis (Figure 4.1).

This model can account for non-stoichiometric production of BCCP-BC complex or CT, whereby if BCCP-BC is overproduced then the N-terminal domain of BCCP is free to autoregulate transcription of the *accBC* gene and if CT is overproduced it binds its own mRNA preventing translation of CT. Therefore, acetyl-CoA levels directly control fatty acid synthesis in *E. coli*.

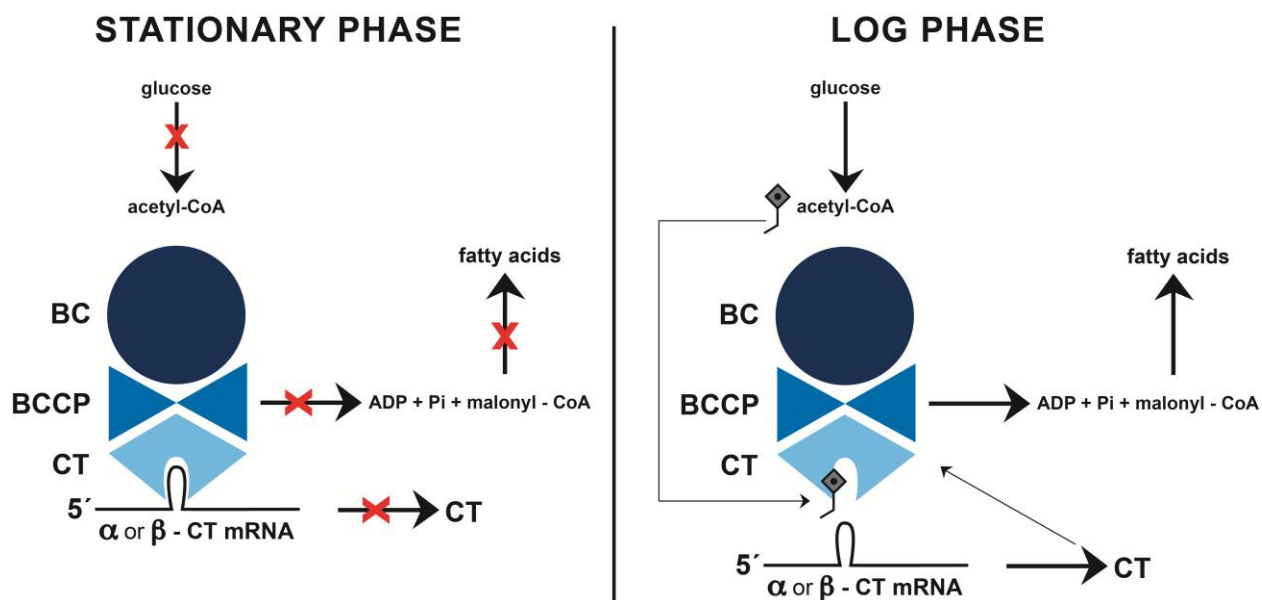


Figure 4.1 Regulatory model of the acetyl-CoA carboxylase complex.

### Future Directions

The elucidation of the ACC complex and the structure of the BCCP-BC complex have generated several new avenues to study this macromolecular enzyme. The methods of purifying and assaying the ACC complex in Chapter 2 allows for testing of previously studied site-directed mutants of the individual subunits in the ACC complex. It will be interesting to see the extent of communication between the BC and CT active sites. For example, do the active site residues His 209, Lys159, and Lys116, which are important for binding ATP in BC, have an effect on the

binding of acetyl-CoA to the complex (Sloane *et al.*, 2001)? These active site mutations could provide insight into the mechanism of ACC activation by acetyl-CoA.

Inhibitors of BC and CT, such as 2-aminooxazole dibenzylamide and andrimid, respectively, were shown to have a synergistic effect on inhibition of the ACC complex. This substantiates the possibility that a dual-ligand inhibitor could inhibit ACC better than either molecule alone by tethering the molecule in proximity to the complex at two anchor points (the active sites) and locking the complex together in an inactive state. Furthermore, a dual-ligand inhibitor linking two BC inhibitors together with a short distance inbetween could inhibit BC activity as well as cause BCCP-BC complexes to aggregate before ACC is formed.

The BCCP-BC complex was unbiotinylated and it would be interesting to know if upon biotinylation, the BCCP molecules change positions on BC to a spot more favorable for catalysis and if the affinity for the CT subunit increases. Also, how does BCCP bind to the BC active site? Both questions first require the overexpression of BCCP and BC in the presence of biotin and separation of the biotinylated complex from the apo form. Subsequently, crystallization studies of the biotinylated complex cocrystallized with ATP, ADP, or carboxyphosphate analogs could provide the answer to both questions.

The subunit stoichiometry of the ACC complex remains elusive. The BCCP-BC complex shed some light on the stoichiometry and suggests a 1:1:1 arrangement of monomers of BC, BCCP, and each CT subunit. This is in agreement with the subunit organization in *Pseudomonas citronellolis* ACC (Choi-Rhee and Cronan, 2003; Fall, 1976) and the subunit and domain organization of the structures of propionyl-CoA carboxylase (Huang *et al.*, 2010) and methylcrotonyl-CoA carboxylase (Huang *et al.*, 2012). Furthermore, pyruvate carboxylase and

eukaryotic ACC express each subunit of *E. coli* ACC as a single domain on each polypeptide chain, which is also suggestive of a 1:1:1 ratio. However, the answer to this question will have to await the crystal structure of the ACC complex or further studies via methods like analytical ultracentrifugation or cryo-electron microscopy.

The crystal structure of the BCCP-BC complex provides three unique interface targets to disrupt formation of the complex. The interface targets could prove useful in drug development considering the current difficulties in developing new and clinically relevant antibiotics targeting the BC and CT active sites (Freiberg *et al.*, 2006; Katz *et al.*, 2006; Mochalkin *et al.*, 2009). Mutagenic analysis of interface residues will delineate which residues are important in complex formation. Then, creation of short peptide sequences or secondary structural elements that mimic the various interfaces could be used to try to disrupt the complex. This technique has been successfully used to disrupt the interaction between Bak (a protein that initiates apoptosis) and Bcl-2, a protein commonly expressed in cancer cells that binds Bak and prevents apoptosis (Chin and Schepartz, 2001). In an attempt to develop a novel anticancer agent Chin and Schepartz (2001) created a miniature protein using Bak interface residues grafted on avian pancreatic protein, which bound 100-fold tighter to Bcl-2 than Bak (Fletcher and Hamilton, 2006). Small molecule interface inhibitors of the BCCP-BC complex interfaces, similar to the Bak-Bcl-2 studies, could yield novel lines of antibiotics to prevent initiation of fatty acid synthesis, which is greatly needed to combat the rising drug resistant microbes.

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**APPENDIX**  
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## VITA

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