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Investigating the Overexpression of and Molecular Mechanism Utilized by Acetyl-CoA Carboxylase

Blaire Peterson

Investigating the Overexpression of and Molecular Mechanism Utilized by Acetyl-CoA Carboxylase

by

Blaire Peterson

Undergraduate honors thesis under the direction of

Dr. Grover Waldrop

Department of Biochemistry

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List of abbreviations

Acetyl-CoA Carboxylase (ACC)

Adenosine Triphosphate (ATP)

Biotin Carboxylase (BC)

Biotin Carboxyl Carrier Protein (BCCP)

Carboxyl Transferase (CT)

Holoenzyme Acetyl-CoA Carboxylase (Holo-ACC)

Isopropyl Beta-D-1-thiogalactopyranoside (IPTG)

Mutated Carboxyl Transferase (αG206V CT)

N-terminal Domain of BCCP (NTD)

Abstract

Acetyl-CoA carboxylase (ACC) is often investigated as a potential target for antibiotics because it is essential to the life of bacteria due to its involvement in fatty acid synthesis and, consequently, membrane biogenesis. ACC is composed of biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). These subunits form a multiprotein complex in which intricate protein-protein interactions are responsible for controlling its activity. Because the two active sites of BC and CT do not interact, there must be a mechanism in which both subunits are regulated in conjunction with the other. There is mounting evidence that the N-terminal domain of BCCP is responsible for the inhibition of BC when acetyl-CoA is absent, but the exact molecular mechanism by which this occurs is unknown. Various procedures for the overexpression of BC and αG206V CT were investigated and the most efficient overexpression method for both enzymes was found. Multiple enzymatic assays were utilized to investigate the molecular mechanism employed by ACC. It was found that turnover of acetyl-CoA by CT is not necessary for the inhibition of BC to be released; instead, acetyl-CoA only needs to be bound to CT. Furthermore, a theory about the reasoning for malonyl-CoA's binding to BCCP was disproved. Future studies should aim to understand how acetyl-CoA binding to CT causes BCCP to release its inhibition of BC.

Background

The regulation of enzymatic activity plays a central role in metabolism in all organisms. Of the many metabolic pathways, fatty acid biosynthesis is one of the most essential throughout the entire biological world (1). The products of this process are crucial to organismal survival for many reasons; they play a major role in energy storage, protein modification, and, most importantly for this thesis, membrane biogenesis (2). The ability to synthesize and alter cellular membranes is an immediate need for bacteria, specifically, because they are unicellular organisms (2). Therefore, maintaining the cell membrane is critical in bacteria because it allows for protection against environmental threats, restricted permeability to solutes, preservation of organismal homeostasis, and other crucial membrane-associated functions (3). Due to the necessity of membrane biogenesis, the fatty acid pathway is an excellent target for novel antibiotics against any species of bacteria that cannot utilize extracellular fatty acids (2).

One enzyme that is often investigated as a potential target for novel antibacterial therapeutics is acetyl-CoA carboxylase (ACC), which catalyzes the first committed step in fatty acid biosynthesis via two half-reactions (Figure 1) (4). Bacterial ACC is a multienzyme complex that consists of the homodimeric biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and an $\alpha_2\beta_2$ heterotetrameric carboxyltransferase (CT) (4). In the first half reaction, BC catalyzes the substrate level phosphorylation of bicarbonate to create a carboxyphosphate intermediate (1). This molecule is reactive and transfers the carboxyl group to biotin, which is covalently bonded to BCCP (1). In the second half reaction, CT catalyzes the transfer of the carboxyl group from biotin to acetyl-CoA; this produces malonyl CoA (1).

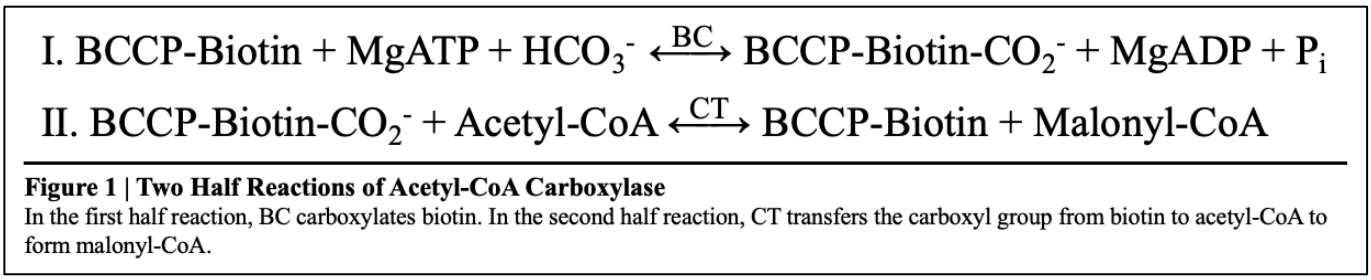

Figure 1 | Two Half Reactions of Acetyl-CoA Carboxylase
In the first half reaction, BC carboxylates biotin. In the second half reaction, CT transfers the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA.

ACC is present in both humans and bacteria, so to create an effective antibiotic, it is essential that the chosen inhibitor is highly selective for bacterial ACC (2). Structural biology is important to the design of potential inhibitors. Much is understood about the biological significance of ACC, but there is a great deal of unanswered questions concerning the molecular mechanism utilized by ACC and how its structure leads to its function.

Regarding what is known about the regulation of bacterial ACC, recent studies have revealed that ACC forms a multiprotein complex in which intricate protein-protein interactions are responsible for controlling its activity (1). ACC uses a two-site ping-pong kinetic mechanism, meaning that the active site for BC and the active site for CT are different and separate; they do not directly interact at all (1). The use of this kinetic mechanism is further confirmed by the fact that both BC and CT, when assayed in isolation, remain enzymatically active when free biotin is present (1). Because the two active sites of BC and CT do not interact, there must be a mechanism in which both subunits are regulated in conjunction with the other. First, CT has a zinc ribbon motif on its beta subunit that allows it to bind to the mRNAs that code for its alpha and beta subunits (5). When bacteria are in stationary phase growth, and there is little acetyl-CoA present, CT will bind to its own mRNA to prevent further translation and enzymatic activity (5). This is important because bacteria only use fatty acid synthesis for membrane biogenesis, so they have no purpose for fatty acids when not replicating. When bacteria are in log phase growth, however, the abundance of

acetyl-CoA outcompetes the mRNA and binds to CT, allowing for catalysis and increased protein production (5) (Figure 2). This negative feedback system allows CT to regulate its own rate of catalysis and translation (1).

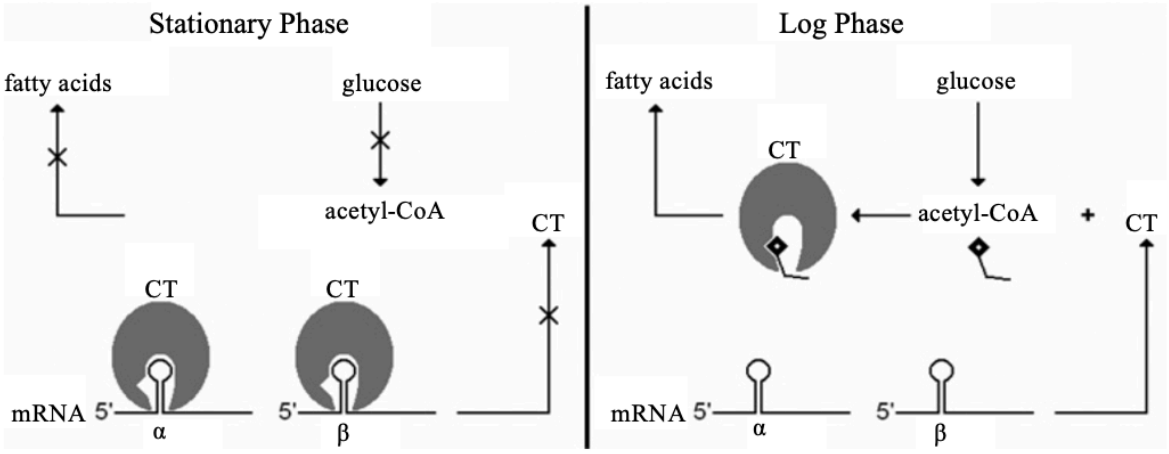

Figure 2 | Regulation of CT
CT regulates itself via binding of its own mRNA. Figure retrieved from Meades Jr., G., Cai, X., Thalji, N.K., Waldrop, G.L., & de Queiroz, M. (5).

If CT regulates itself, then the question remains regarding how BC is regulated. The level of intracellular ATP in *Escherichia coli* is 1-5mM, while the Km for ATP for BC is 0.081mM (6, 7). Using a derivation of the Michaelis-Menten equation, it was determined that BC is always nearly saturated with substrate (Figure 3). However, it would be incredibly wasteful if BC were to continuously hydrolyze ATP, so there must be a mechanism that inhibits its enzymatic activity (1). It turns out that, when BC is assayed in isolation, biotinylated BCCP is not a sufficient substrate (Figure 4) (4). Furthermore, BC

$$\frac{V}{Vmax} = \frac{\frac{[S]}{Km}}{1 + \frac{[S]}{Km}} \qquad \frac{V}{Vmax} = 0.974$$

Figure 3 | Mathematical Demonstration of the Saturation of BC
When V/Vmax = 1, the enzyme is completely saturated with substrate. Using [S] = 2mM and Km = 0.081mM, the V/Vmax for BC with ATP is 0.974. BC is nearly completely saturated with ATP in a bacterial cell.

has low activity even when all subunits of ACC are present (Figure 4) (4). Of all the assays conducted by Broussard, T.C., *et al.*, the only one in which BC showed significant activity was when CT, biotinylated BCCP, and acetyl-CoA were present (Figure 4) (1). This indicates that something is interacting with BC in the multiprotein complex to inhibit it when acetyl-CoA is not present; clearly, acetyl-CoA must be bound to CT for BC to hydrolyze ATP (1).

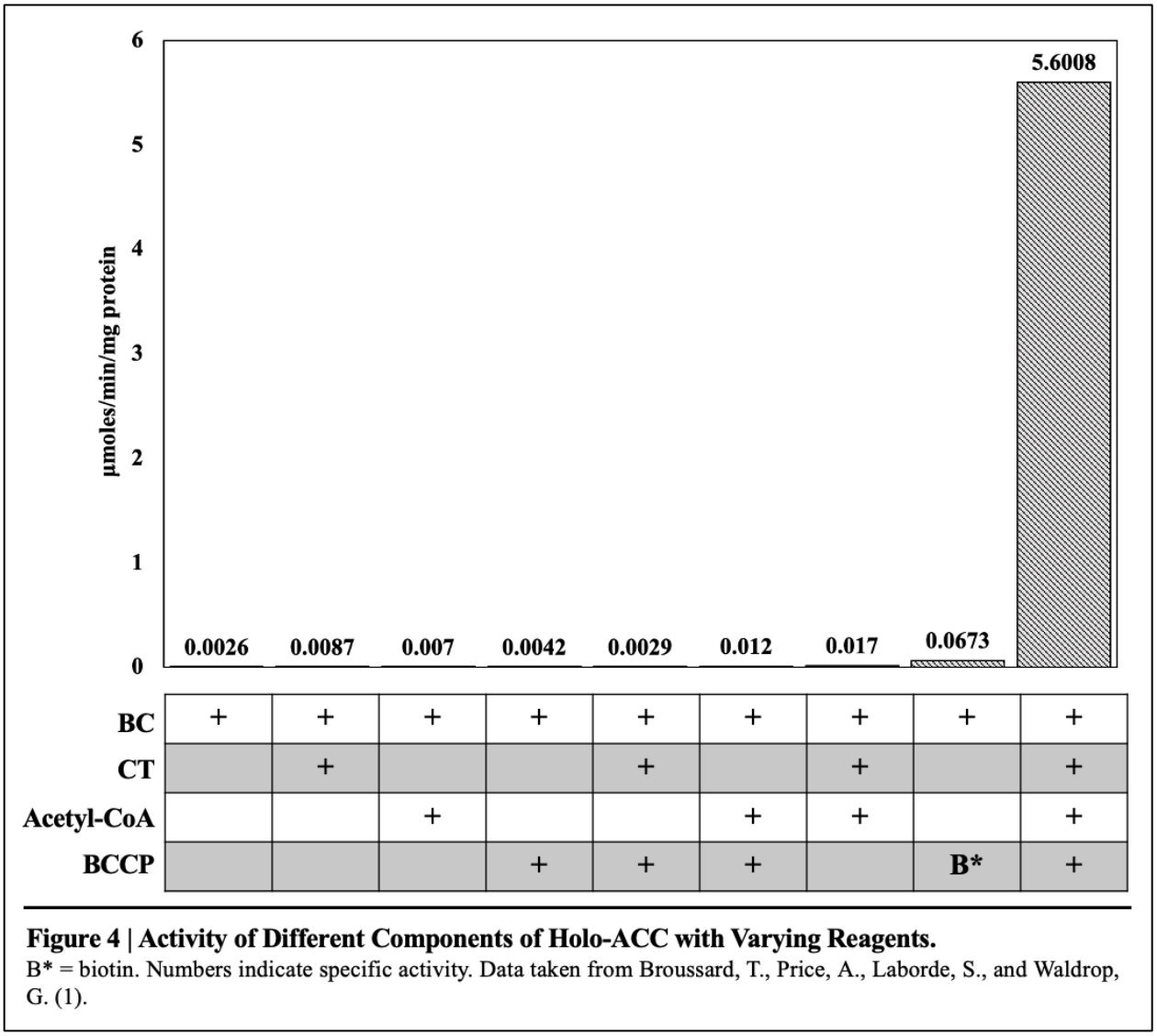

Figure 4 | Activity of Different Components of Holo-ACC with Varying Reagents.
B* = biotin. Numbers indicate specific activity. Data taken from Broussard, T., Price, A., Laborde, S., and Waldrop, G. (1).

One proposed mechanism of inhibition is that the N-terminal domain of BCCP (NTD) inhibits the activity of BC when acetyl-CoA is not present (1). This conclusion was drawn based on the fact that, when the biotinylated C-terminal domain of BCCP is used as a substrate for BC in the absence

of CT and acetyl-CoA, the activity of BC was significant (1). NTD seems to play a regulatory role to prevent non-productive hydrolysis of ATP. While there is mounting evidence that the N-terminal domain of BCCP is responsible for the inhibition of BC when acetyl-CoA is absent, it is still unclear whether turnover of acetyl-CoA is needed to hydrolyze ATP. The work in this thesis addresses this question and further investigates the molecular mechanism employed by acetyl-CoA carboxylase.

Materials and methods

Reagents

Amicon ultracentrifugal filters were from Millipore. Enzymatic assays were performed using a 60-UV-Vis Spectrophotometer from Agilent Technologies. IPTG was from Gold Biotechnology. The Ni-NTA Superflow Cartridge column was purchased from Qiagen. Magnesium chloride and potassium chloride were purchased from Fischer Scientific. All other reagents were from Sigma-Aldrich. The expression plasmid containing biotin carboxylase with a His tag at the N-terminus was previously constructed and transformed into *E. coli* as described by Blanchard *et al.* (7). The expression plasmid containing carboxyltransferase with a His tag at the N-terminus of the α-subunit was previously constructed and transformed into *E. coli* as described by Blanchard and Waldrop (8). Biotinylated BCCP with a His tag on the N-terminus was a gift from Pfizer (1). The catalytically inactive mutant of CT (αG206V) was created by site-directed mutagenesis as previously described (5).

Expression of BC, CT, and αG206V CT

The overexpression strain for BC was streaked on an LB agar plate containing 50 μg/ml ampicillin. A single colony was used to inoculate 0.5L of LB medium containing 50ug/mL ampicillin in a 2L

flask. The cultures were shaken at 160 rpm at 37 °C until midlog phase, at which time 200 μM IPTG was added to induce gene expression. The rate of shaking was lowered to 120 rpm, the temperature was lowered to 25 °C, and the cultures were incubated overnight. The cells were harvested by centrifugation at 8,000 g for 15 minutes at 4°C. The supernatant was decanted, and the pellet was resuspended in wash buffer, or NPI-10 [10mM Hepes-NaOH, 500mM NaCl, 10mM imidazole (pH 8.0)]. CT and αG206V CT were expressed in the same manner, except 50μM of IPTG was used for induction.

Purification of BC, CT, and αG206V CT

Nickel affinity chromatography was used for the purification of all proteins. The resuspended cells were lysed by sonication and 0.1 U/ml DNase was added to remove nucleic acids. The cells were centrifuged at 17,096g for 1 hour at 4°C. The supernatant was removed and run through a nickel column pre-equilibrated with wash buffer. The column was washed with 60 mL of NPI-10 followed by 30 mL of a second wash buffer, or NPI-30 [10mM Hepes-NaOH, 500mM NaCl, 30mM imidazole (pH 8.0)]. The protein was eluted from the column using an elution buffer [10mM Hepes-NaOH, 500mM NaCl, 250mM imidazole (pH 8.0)] and 10 1 mL fractions were collected. The fractions containing BC, CT, or αG206V CT were identified using SDS-PAGE (Figure 5). All appropriate fractions were pooled; the protein was then dialyzed against a buffered EDTA solution [10mM Hepes-KOH, 150mM KCl, 1mM EDTA (pH 8.0)] at 4 °C for 24 hours to remove any residual nickel, followed by dialysis against a buffered storage solution [10mM Hepes-KOH, 150mM KCl (pH 8.0)] at 4 °C for 24 hours to remove any residual EDTA. The protein was then concentrated, aliquoted, and stored at −80 °C.

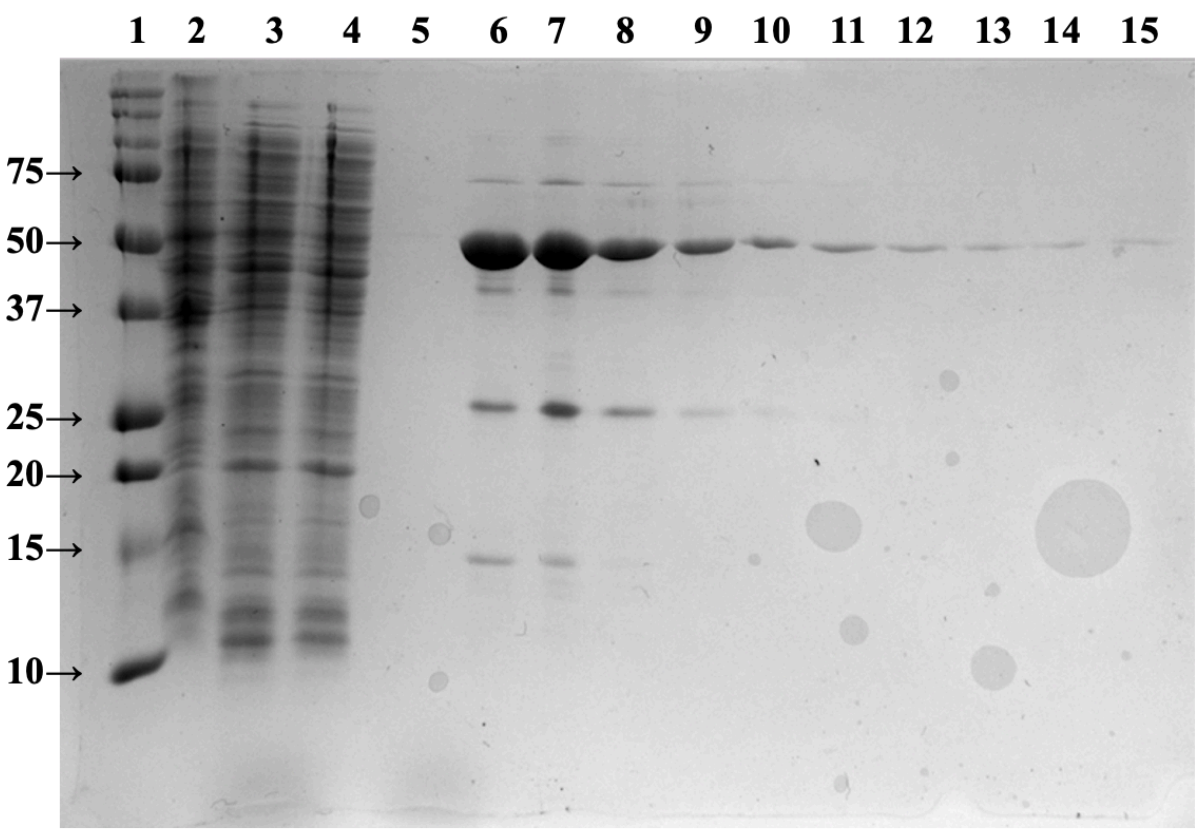

Figure 5 | SDS-PAGE of BC
Numbers on top correspond to the sample used within the lane: (1) Molecular weight standards, (2) Whole cell lysate, (3) Initial load flow through, (4) NPI-10 wash flow through, (5) NPI-30 wash flow through, (6-15) Eluted BC fractions. The kilodaltons of the molecular weight standards are given on the left.

Enzymatic assays

- Activity of CT

The activity of carboxyltransferase was determined spectrophotometrically by measuring the production of reduced nicotinamide adenine dinucleotide (NADH) (Figure 6). CT was assayed in the non-physiological direction; this assay utilizes CT and two enzymes from the citric acid cycle, citrate synthase (CS) and malate dehydrogenase (MDH), to get a quantifiable product. CT decarboxylates malonyl-CoA to form acetyl-CoA (Figure 6). MDH oxidizes malic acid and reduces NAD+ to form oxaloacetate and NADH. CS uses oxaloacetate from MDH and acetyl-

CoA from CT to make citrate, which is a dead-end product. The standard free energy change ($\Delta G^{\circ\prime}$) for MDH is +27.70 kJ/mol, meaning that equilibrium lies far towards malic acid (9). On the other hand, the $\Delta G^{\circ\prime}$ for CS is -36.43 kJ/mol, so equilibrium for that reaction lies towards citrate (9). For MDH to produce oxaloacetate, oxaloacetate needs to be removed from solution by CS. As acetyl-CoA is produced and subsequently used to remove oxaloacetate, MDH produces more oxaloacetate and, by consequence, NADH. A spectrophotometer is then used to measure the production of NADH because NADH absorbs ultraviolet light at 340 nm.

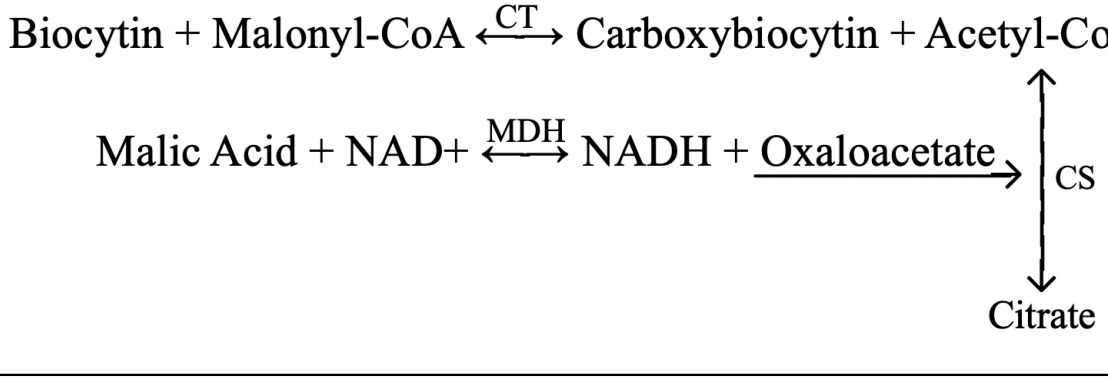

Figure 6 | Schematic of Carboxyltransferase Assay
The activity of CT was determined spectrophotometrically by measuring the production of NADH at 340nm.

Each reaction mixture contained 0.5 mM NAD+, 0.6 mg/mL bovine serum albumin, 0.1 mM malic acid, 0.12 mg/mL MDH, 0.05 mg/mL CS, 100 mM Hepes (pH 7.8), 0.04 mM malonyl-CoA, 6 mM of biocytin, and water for a total amount of 0.5 mL. Biocytin, an analogue of biotin, was used rather than biotin because it was discovered that it is a better substrate for CT (9). Biocytin is biotin with a lysine attached to the valeric side chain (Figure 7). All reactions took place in a 1 cm path-length quartz cuvette and were initiated by the addition of CT to the reaction mixture. Data was collected using an Agilent UV-Vis spectrophotometer and a data acquisition program. Each assay was made in triplicates, and the mean and standard error were reported.

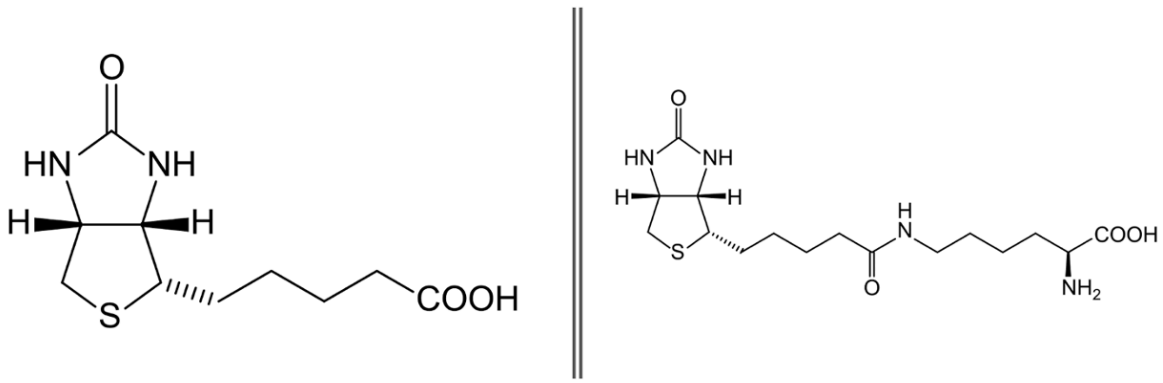

Figure 7 | Biotin vs Biocytin
Biocytin, an analogue of biotin, contains lysine attached to the valeric side chain.

- Activity of Holo-ACC

The activity of the holoenzyme acetyl-CoA carboxylase (holo-ACC) was determined spectrophotometrically by following the oxidation of NADH at 340 nm. This assay utilizes holo-ACC, pyruvate kinase (PK), and lactate dehydrogenase (LDH) to get a quantifiable product. BC hydrolyzes ATP and produces ADP. PK uses that ADP and phosphoenolpyruvate (PEP) to produce ATP and pyruvate. LDH catalyzes the formation of lactate and NAD+ from pyruvate and NADH (Figure 8). A spectrophotometer is then used to measure the decrease of NADH.

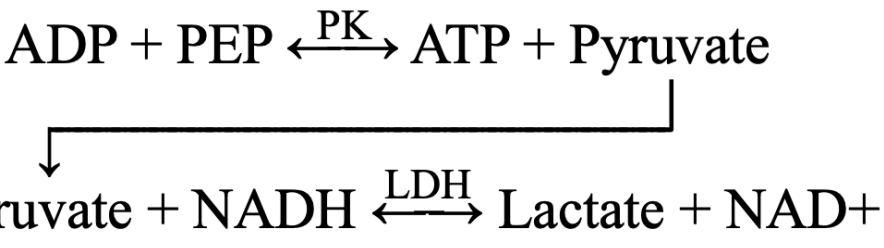

Figure 8 | Schematic of Acetyl-CoA Carboxylase Assay
The activity of Holo-ACC was determined spectrophotometrically by following the production of ADP using the coupled enzyme assay shown above and measuring the decrease of NADH at 340 nm.

Each reaction mixture contained 10.5 units of pyruvate kinase, 17.5 units of lactate dehydrogenase, 0.5 mM PEP, 0.2 mM NADH, 20 mM $MgCl_2$, 15 mM potassium bicarbonate, 100 mM Hepes (pH

7.8), 0.2 mM ATP, and water for a total amount of 0.5 mL. For the first experiment involving holo-ACC, each reaction also contained 0.5 mM acetyl-CoA. For the second experiment involving holo-ACC, each reaction also contained 0.5 mM acetyl-CoA, 1 mM malonyl-CoA, 2 mM malonyl-CoA, or neither reagent. All reactions took place in a 1 cm path-length quartz cuvette and were initiated by the addition of holo-ACC to the reaction mixture. Data was collected using an Agilent UV-Vis spectrophotometer and a data acquisition program. Each assay was made in triplicates or duplicates and the mean and standard error were reported.

- Activity of BC/BCCP

The activity of biotin carboxylase and biotinylated biotin carboxyl carrier protein (BC/BCCP) was measured using the same assay as used for holo-ACC. Each reaction mixture contained 10.5 units of pyruvate kinase, 17.5 units of lactate dehydrogenase, 0.5 mM PEP, 0.2 mM NADH, 20 mM $MgCl_2$, 15 mM potassium bicarbonate, 100 mM Hepes (pH 7.8), 0.2 mM ATP, and water for a total amount of 0.5 mL. Each reaction also contained 0.5 mM acetyl-CoA or 1 mM malonyl-CoA. All reactions took place in a 1 cm path-length quartz cuvette and were initiated by the addition of BC/BCCP to the reaction mixture. Data was collected using an Agilent UV-Vis spectrophotometer and a data acquisition program. Each assay was completed once.

Results and discussion

Overexpression of BC

To optimize the overexpression of BC, various procedures for BC production were investigated. The overexpression system for BC employs the plasmid pET14b with a lac operon. This lac operon controls the gene that codes for BC and can be induced by the addition of IPTG. Different concentrations of IPTG were added to separate *E. coli* growths (0.5L) to determine which

concentration produced the most BC. Furthermore, each culture of *E. coli* with varying concentrations of IPTG were grown at either 4°C or 37°C over a period of 3 or 1 days, respectively, to determine the best temperature conditions. SDS-PAGE was used to visualize the amount of protein produced by each culture, and the band intensities were measured using IMAGEJ. The relative amount of protein produced by each growth are shown in Figure 9. It was determined that the best growth conditions for overexpressing BC were 37 °C with 200µM IPTG.

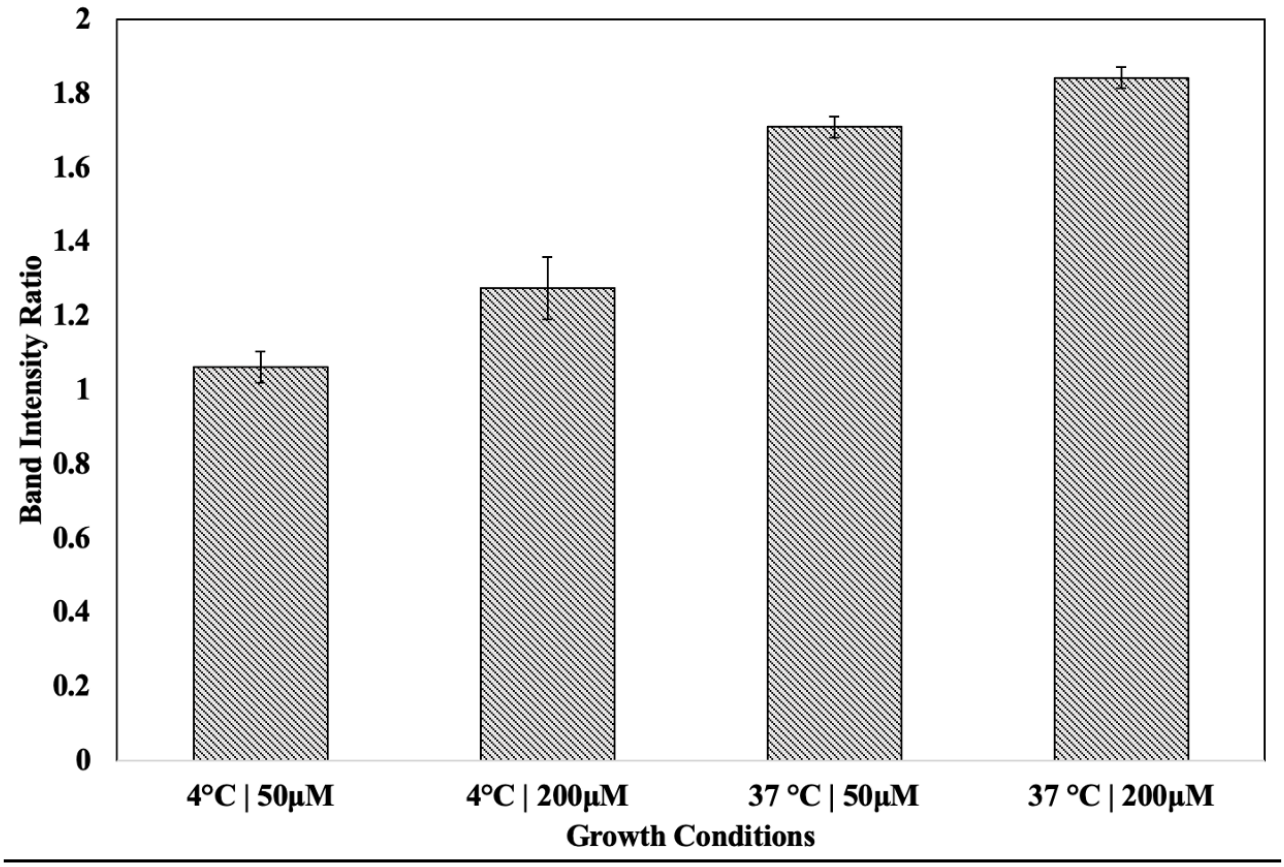

Figure 9 | BC Produced under Varying Conditions
SDS-PAGE was used to visualize the amount of protein produced by each condition and the band intensities were measured using IMAGEJ. The concentration of IPTG for each condition is shown.

Overexpression of αG206V CT

The genes coding for the α and β subunits of the mutant CT are, like BC, in plasmid pET14b and controlled by the lac operon, so expression can be induced with IPTG. To optimize the overexpression of αG206V CT, different concentrations of IPTG were tested and the most effective amount was determined. SDS-PAGE was used to visualize the amount of protein produced by each culture, and the band intensities were measured using IMAGEJ (Figure 10). The relative amount of protein produced by each concentration of IPTG is show in Figure 11. 50μM of IPTG produced the most αG206V CT. The wildtype CT was produced using the same conditions as the mutant.

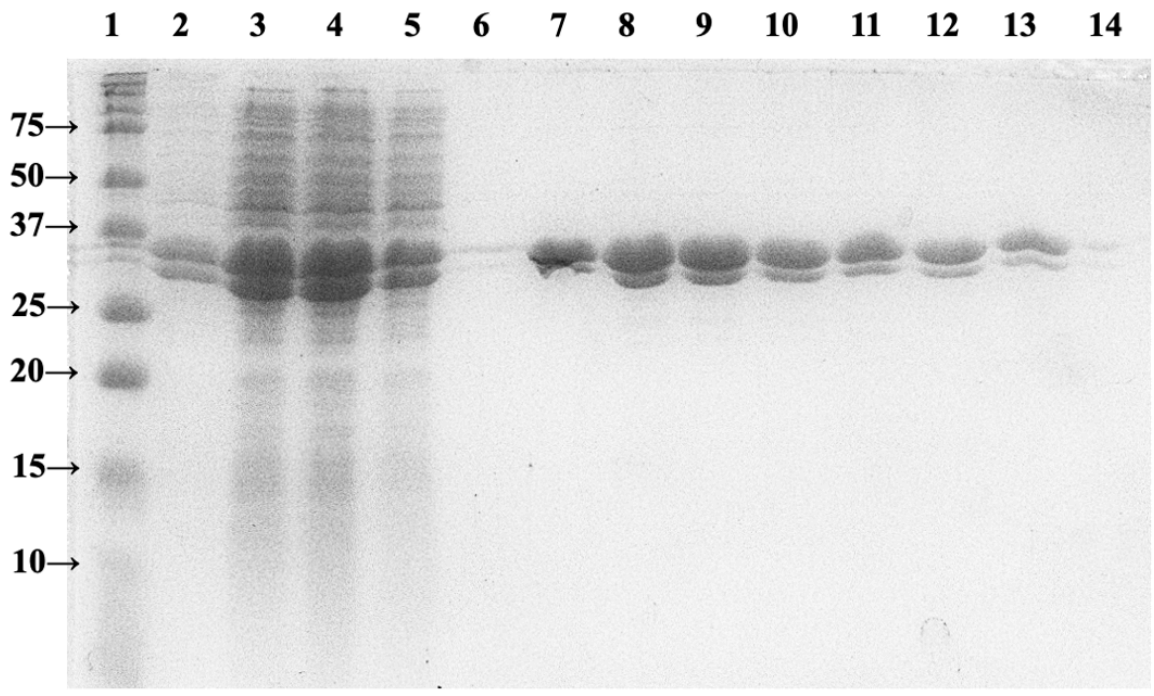

Figure 10 | SDS-PAGE of αG206V CT
Numbers on top correspond to the sample used within the lane: (1) Molecular weight standards, (2) Cell pellet, (3) Whole cell lysate, (4) Initial load flow through, (5) NPI-10 wash flow through, (6) NPI-30 wash flow through, (7-14) Eluted αG206V CT fractions. The kilodaltons of the molecular weight standards are given on the left.

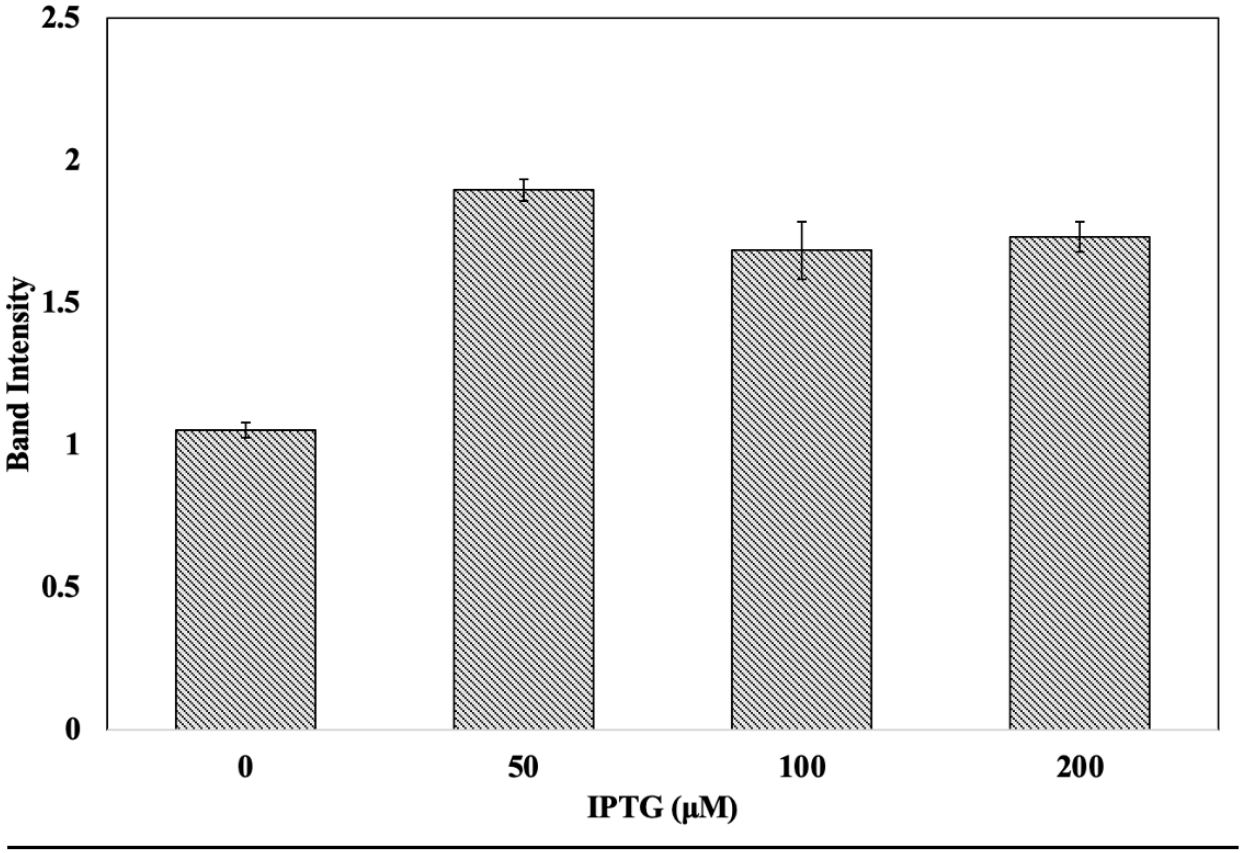

Figure 11 | αG206V CT Produced Under Varying Amounts of IPTG
SDS-PAGE was used to visualize the amount of protein produced by each condition and the band intensities were measured using IMAGEJ.

Wildtype CT vs αG206V CT

To confirm that αG206V CT was inactive, CT and αG206V CT were assayed in the reverse-physiological direction as previously described (Materials and Methods). The initial velocities of each reaction were measured, and the mean and standard error were calculated. From these values, the control, wildtype CT, was designated as 100% activity, while the mutant CT was compared proportionally. As shown in Figure 12, αG206V CT was completely inactive.

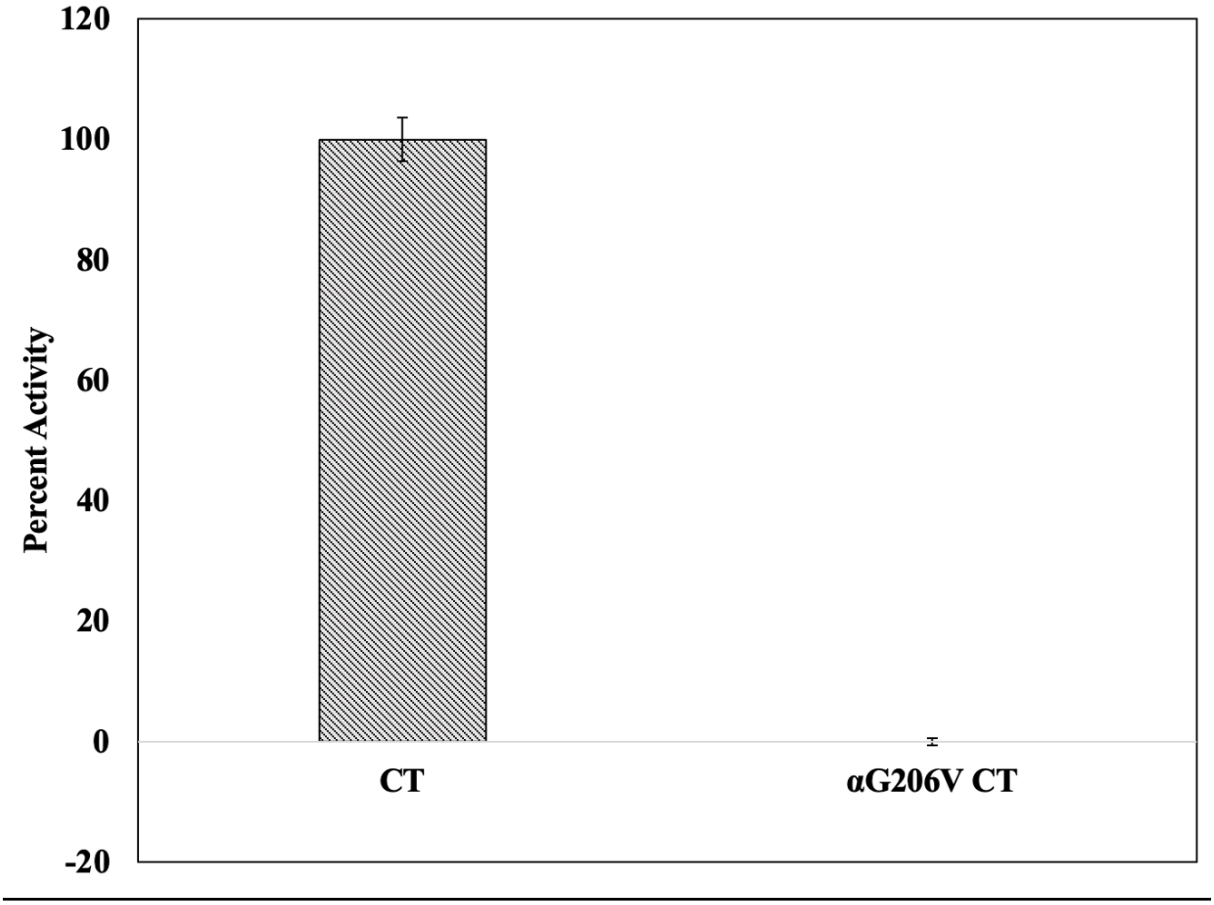

Figure 12 | Activity of WT CT vs αG206V CT
Each assay was done in triplicate. Initial velocities were measured, and the mean and standard error were calculated. The control, CT, was set to 100% activity, and the mutant was compared proportionally.

Holo-ACC vs Mutant Holo-ACC

Once it was confirmed that αG206V CT was inactive, holo-ACC, both wildtype and mutant, were assembled using a 1:2:1 ratio of BC:BCCP:CT. The activity of the holo-ACC enzyme was assayed by observing the oxidation of NADH as previously described (Materials and Methods). The initial velocities were measured for each run, and the mean and standard deviation were calculated. From those values, the control, holo-ACC, was designated as 100% activity, and the mutant holo-ACC was compared proportionally. As shown in Figure 13, the mutant holo-ACC was found to be active, indicating that turnover of acetyl-CoA is not needed for the enzyme complex to function.

Instead, the data indicates that acetyl-CoA only needs to be bound to the β subunit of CT for NTD to release its inhibition of BC. It is known that αG206V CT binds acetyl-CoA because acetyl-CoA binds to the β subunit, while the mutation that renders the enzyme inactive is in the α subunit (6).

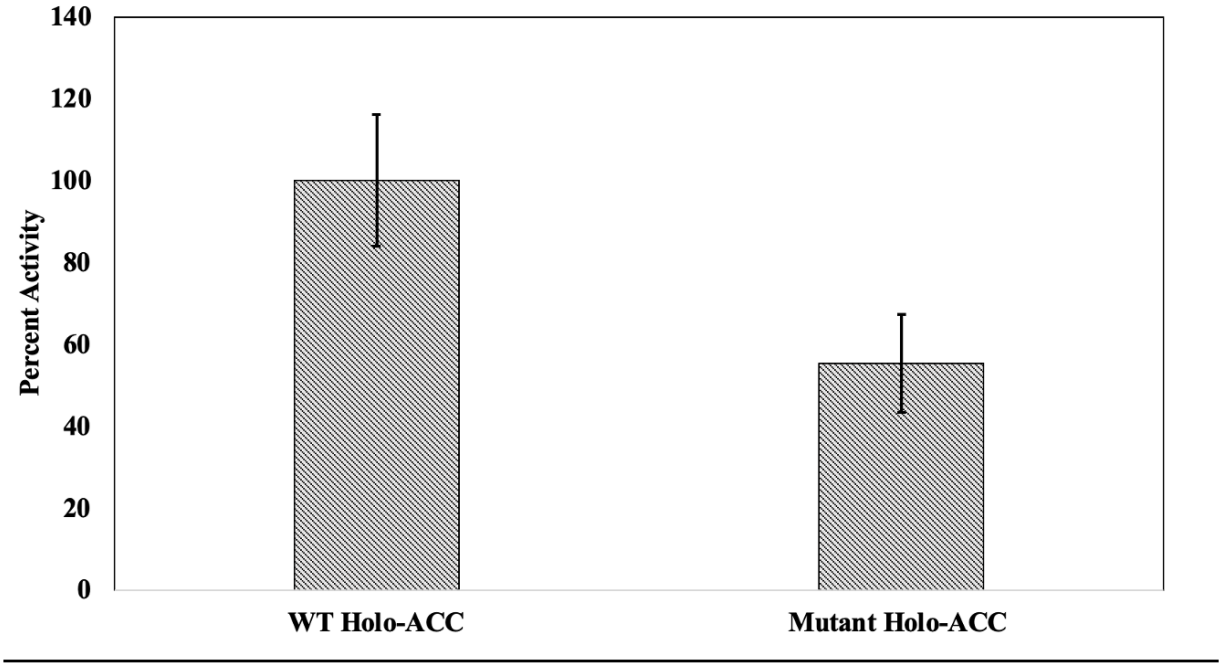

Figure 13 | Activity of Holo-ACC vs Mutant Holo-ACC
Each assay was done in triplicate. Initial velocities were measured, and the mean and standard error were calculated. The control, WT Holo-ACC, was set to 100% activity, and the mutant was compared proportionally.

The mutant was clearly active; however, it had 55.34% the activity of wildtype holo-ACC. This creates a new question about the molecular mechanism of ACC. Clearly, the binding of acetyl-CoA is essential for the activity of the enzyme, but is there another regulatory component that allows for an increased amount of activity? One theory that was posited based on this data was that as acetyl-CoA is carboxylated and malonyl-CoA is formed, malonyl-CoA feeds back and binds NTD to further reduce its inhibition of BC. This theory stems from previous data that has proven that malonyl-CoA binds to NTD; inhibition studies were conducted to discover which substrates bind to NTD, and it was found that biotin, biocytin, malonyl-CoA, and ATP bind it

competitively (Table 1). A reason for this interaction has yet to be discovered, and malonyl-CoA creating a positive feedback loop was postulated as a possibility.

Table 1 | Unpublished data from inhibition studies completed by Alexandra Evans, Waldrop Lab

First Half Reaction (BC + Biotin)

Inhibitor (CT + Biotin)	Km vs Biocytin (mM)	Ki vs Biocytin	Type of inhibition	Ki vs Malonyl-CoA	Km vs Malonyl-CoA	Type of Inhibition
NTD μM	19.7 ± 3.3	119.8 ± 13.9	Competitive	150.0 ± 25.6	47.1 ± 5.2	Competitive

Second Half Reaction (CT + Biocytin)

Inhibitor (BC + Biotin)	Km vs Biotin (nM)	Ki vs Biotin	Type of Inhibition	Ki vs ATP	Km vs ATP (μM)	Type of Inhibition
NTD μM	53.9 ± 6.3	218.1 ± 29.8	Competitive	34.5 ± 6.2	1.4 ± 0.2	Competitive

Holo-ACC and Malonyl-CoA

To determine if malonyl-CoA plays a part in releasing the inhibition of BC, assays were run with wildtype holo-ACC and one of the following: 0.5mM acetyl-CoA, 1mM malonyl-CoA, 2mM malonyl-CoA, or neither reagent. Holo-ACC was assembled in a 1:6:1 ratio of BC:BCCP:CT because it was discovered that this ratio gave a higher level of activity. The initial velocities were measured for each run and the mean and standard deviation were calculated. From those values, the control, holo-ACC with 0.5mM acetyl-CoA, was designated as 100% activity, and the other assays were compared proportionally. It is important to note that previous data has shown that CT does not catalyze its reaction in the reverse-physiological direction when a part of the ACC complex, so there was no way for acetyl-CoA to be formed in the reactions containing only malonyl-CoA or neither reagent (1). As shown in Figure 14, there is virtually no activity in the absence of both acetyl-CoA and malonyl-CoA, but there is activity in the presence of only malonyl-CoA, albeit it is much less activity than shown with acetyl-CoA.

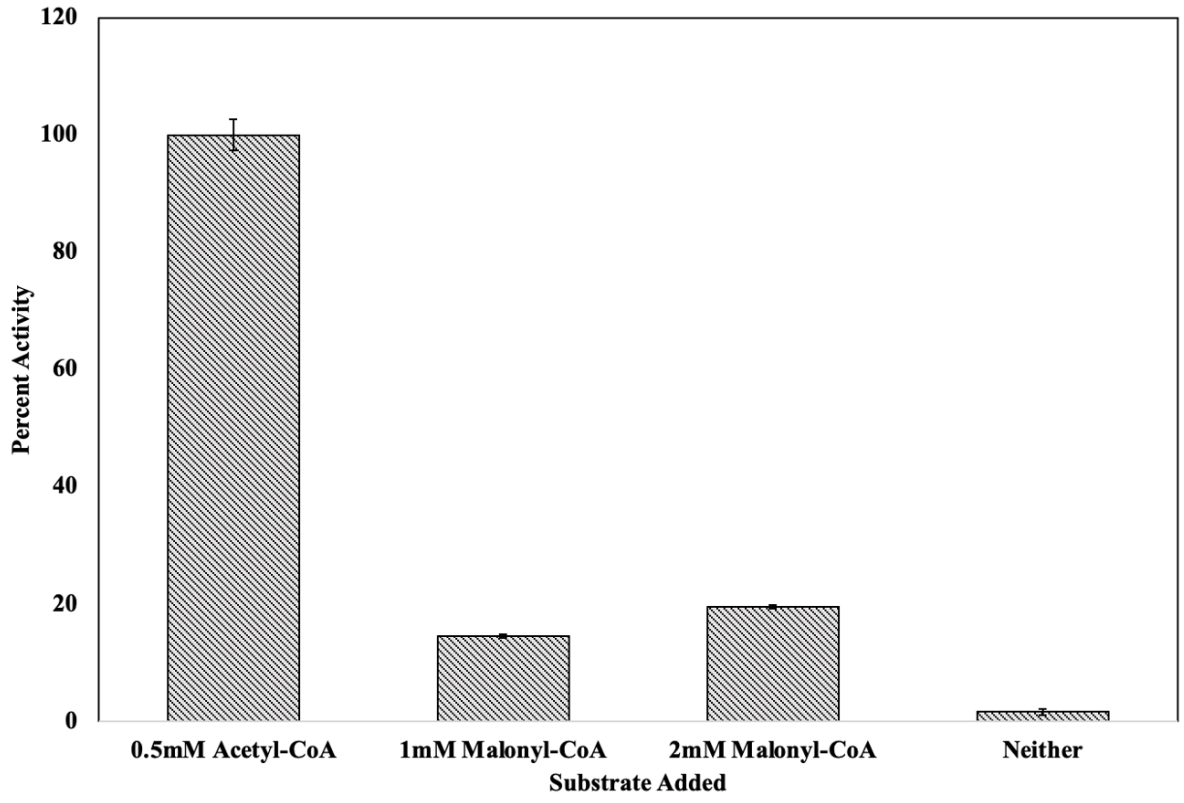

Figure 14 | Activity of Holo-ACC with Various Substrates
Each assay was done in duplicate. Initial velocities were taken, and the mean and standard error were calculated. The control, 0.5mM Acetyl-CoA, was set to 100% activity, and the other assays were compared proportionally.

BC/BCCP and Malonyl-CoA

The data presented in Figure 14 could indicate that malonyl-CoA does in fact bind to BCCP to partially release its inhibition of BC, but it could also result from malonyl-CoA binding to the acetyl-CoA binding site on CT. To test this, assays with only BC/BCCP were run. The first assay included BC/BCCP with no malonyl-CoA, and the second assay included BC/BCCP and 1mM malonyl-CoA. The initial velocities were measured. It was found that neither condition produced activity.

This data indicates that malonyl-CoA does not use a feedback system and was merely binding to CT in the previous experiment. However, it does further prove that CT only needs to be bound to acetyl-CoA to cause NTD to release its inhibition of BC because, in the previous experiment, malonyl-CoA was likely binding to the acetyl-CoA binding site on CT and still managed to allow for some activity. This data does beg the question, however, of why NTD binds malonyl-CoA in the first place.

Final Thoughts and Future Studies

Clearly, BC and CT are regulated in conjunction with another, and the main components of regulation are BCCP and acetyl-CoA. BCCP has two functionally significant domains (10). The C-terminal domain has a highly conserved biotin domain, and the three-dimensional structure has been determined through nuclear magnetic resonance (NMR) and crystallography (10). The other domain, the N-terminal domain, is responsible for the interaction with and inhibition of BC (10). Unlike the C-terminal domain, there is no three-dimensional structure, so it has been postulated that it is an intrinsically disordered protein region (IDPR). IDPR's are domains with little conformational stability that continually unfold and refold upon binding of a substrate (11). NTD, specifically, was found to bind multiple substrates that are dissimilar, such as biotin, malonyl-CoA, and ATP (Table 1). The role of IDPR's appears to be, overwhelmingly, regulation because of their flexible nature; this aligns with the role of NTD in acetyl-CoA Carboxylase (11).

Pyruvate carboxylase is an enzyme that is structurally very similar to acetyl-CoA carboxylase. It is also a biotin-dependent carboxylase with a BC, CT, and BCCP component (12). It differs in that it has four identical subunits; each contain a BC, CT, and BCCP region that binds biotin via a

specific lysine residue (12). Because of its similarity to acetyl-CoA carboxylase, pyruvate carboxylase was investigated to identify a potential molecular mechanism for ACC. Regarding the mechanism of pyruvate carboxylase, when acetyl-CoA binds to the CT domain, the entire enzyme undergoes a conformational change that stabilizes its tetrameric structure (13). The binding of acetyl-CoA activates the BC domain where ATP hydrolysis and biotin carboxylation occur; it also enhances the binding of bicarbonate (13). This indicates that acetyl-CoA promotes the formation of the carboxyphosphate immediate by BC (13). Acetyl-CoA is able to relieve the inhibition of BC because its binding domain is located at the interface of the CT and BCCP domains (13). In acetyl-CoA carboxylase, it is already heavily suggested that NTD is responsible for the inhibition of BC, so if acetyl-CoA is somehow binding BCCP along with CT, this could explain how BCCP is able to change its effect on BC when acetyl-CoA binds. The most obvious step for testing this theory would be to do inhibition studies with NTD and acetyl-CoA and determine if NTD binds acetyl-CoA. If it does, then this theory has merit, and an explanation for NTD binding malonyl-CoA could simply be because malonyl-CoA is structurally similar to acetyl-CoA. If not, a new theory will have to be put forward.

Through composing this thesis, a sufficient method of overexpressing BC and αG206V CT was identified and different theories about the molecular mechanism utilized by acetyl-CoA carboxylase were explored. The most important finding of this thesis was that turnover of acetyl-CoA is not needed for holo-ACC to function, although it appears to be needed for holo-ACC to have full activity. Future studies should aim to discover what substrates each subunit of ACC binds, and how those results may reveal the full molecular mechanism.

References

1. Broussard, T. C., Price, A. E., Laborde, S. M., & Waldrop, G. L. (2013). Complex formation and regulation of Escherichia coli acetyl-CoA carboxylase. *Biochemistry*, *52*(19), 3346–3357. https://doi.org/10.1021/bi4000707
2. Polyak, S. W., Abell, A. D., Wilce, M. C., Zhang, L., & Booker, G. W. (2012). Structure, function and selective inhibition of bacterial acetyl-coa carboxylase. *Applied microbiology and biotechnology*, *93*(3), 983–992. https://doi.org/10.1007/s00253-011-3796-z
3. Parsons, J. B., & Rock, C. O. (2011). Is bacterial fatty acid synthesis a valid target for antibacterial drug discovery?. *Current opinion in microbiology*, *14*(5), 544–549. https://doi.org/10.1016/j.mib.2011.07.029
4. Broussard, T. C., Kobe, M. J., Pakhomova, S., Neau, D. B., Price, A. E., Champion, T. S., & Waldrop, G. L. (2013). The three-dimensional structure of the biotin carboxylase-biotin carboxyl carrier protein complex of E. coli acetyl-CoA carboxylase. *Structure (London, England: 1993)*, *21*(4), 650–657. https://doi.org/10.1016/j.str.2013.02.001
5. Meades, G., Cai, X., Thalji, N. K., Waldrop, G. L., & de Queiroz, M. (2011). Mathematical modelling of negative feedback regulation by carboxyltransferase. *IET systems biology*, *5*(3), 220–228. https://doi.org/10.1049/iet-syb.2010.0071
6. Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., & Lu, S. (2013). Release of extracellular ATP by bacteria during growth. *BMC microbiology*, *13*, 301. https://doi.org/10.1186/1471-2180-13-301
7. Blanchard, C. Z., Lee, Y. M., Frantom, P. A., & Waldrop, G. L. (1999). Mutations at four active site residues of biotin carboxylase abolish substrate-induced synergism by biotin. *Biochemistry*, *38*(11), 3393–3400. https://doi.org/10.1021/bi982660a

8. Blanchard, C. Z., & Waldrop, G. L. (1998). Overexpression and kinetic characterization of the carboxyltransferase component of acetyl-CoA carboxylase. *The Journal of biological chemistry*, *273*(30), 19140–19145. https://doi.org/10.1074/jbc.273.30.19140
9. Li, X., Dash, R. K., Pradhan, R. K., Qi, F., Thompson, M., Vinnakota, K. C., Wu, F., Yang, F., & Beard, D. A. (2010). A database of thermodynamic quantities for the reactions of glycolysis and the tricarboxylic acid cycle. *The journal of physical chemistry. B*, *114*(49), 16068–16082.
10. Choi-Rhee, E., & Cronan, J. E. (2003). The biotin carboxylase-biotin carboxyl carrier protein complex of Escherichia coli acetyl-CoA carboxylase. *The Journal of biological chemistry*, *278*(33), 30806–30812. https://doi.org/10.1074/jbc.M302507200
11. Uversky V. N. (2015). Functional roles of transiently and intrinsically disordered regions within proteins. *The FEBS journal*, *282*(7), 1182–1189. https://doi.org/10.1111/febs.13202
12. Tong L. (2013). Structure and function of biotin-dependent carboxylases. *Cellular and molecular life sciences: CMLS*, *70*(5), 863–891. https://doi.org/10.1007/s00018-012-1096-0
13. Adina-Zada, A., Zeczycki, T. N., & Attwood, P. V. (2012). Regulation of the structure and activity of pyruvate carboxylase by acetyl CoA. *Archives of biochemistry and biophysics*, *519*(2), 118–130. https://doi.org/10.1016/j.abb.2011.11.015

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