4-2005

Functional Genomics of Carboxyltransferase

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by

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Undergraduate honors thesis under the direction of

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Submitted to the LSU Honors College in partial fulfillment of
the Upper Division Honors Program.

April, 2005

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Acknowledgements

I owe thanks to the many people who contributed to my research and supported my endeavors. First of all, I would like to thank Dr. Grover Waldrop for mentoring me during the four years I have worked in his laboratory. He has provided me a place to learn and an environment to apply what I have learned. He has shown absolute dedication to both teaching and challenging me, and his support and leadership have not gone unappreciated.

I would like to show my appreciation to graduate student Pat Bilder for his work on the crystal structure of carboxyltransferase, and for helping me make sense of my data. I also owe thanks to graduate student Brian Benson, who provided much of the preliminary work needed to get my research project off the ground. I must also thank fellow students Katie Breaux and Matt Long for their help in the lab and for washing far more dishes than I ever did. Additionally, I would like to thank the members of my thesis committee—Dr. Alan Baumeister, Dr. Yong-Hwan Lee, and Dr. Grover Waldrop—for their time and interest in my research.

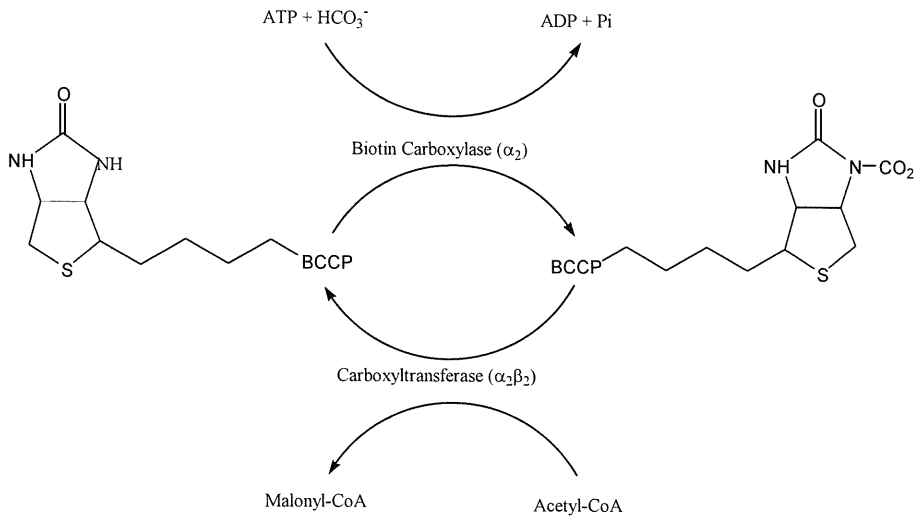
Lastly, and most importantly, I feel the least I can do is thank my parents—Robert and Lisa Ranney—for their continued support over the years. They have provided more than I can ask for, and any successes I have had has been a direct product of their unwavering dedication and commitment to my goals.

Abstract

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in long-chain fatty acid biosynthesis. The *Escherichia coli* form of the enzyme consists of biotin carboxylase, a biotin carboxyl carrier protein, and carboxyltransferase. A newly solved crystal structure of carboxyltransferase provided the basis for several new areas of research. Using the structure and sequence alignments, two residues—K142 and R145 on the alpha subunit of carboxyltransferase—were targeted for site-directed mutagenesis. Kinetic characterization revealed K_m values for malonyl CoA and biocytin were similar to wildtype carboxyltransferase. However, the V_{max} values of the two mutants were found to have decreased activities compared to wildtype. The V_{max} of K142 was 22.7% of the wildtype maximal velocity, and the V_{max} of the R145 was 3.5% of the wildtype value. Both of these residues are not located in the active site of the enzyme, and they are hypothesized to function in stabilizing the interaction between subunits in carboxyltransferase, a heterotetramer. This thesis also investigated the inhibition of carboxyltransferase by the antibiotic tetracycline. Inhibition of carboxyltranferase by tetracycline with respect to malonyl CoA exhibited competitive inhibition with a K_{is} of 0.34±0.06 mM.

Introduction

Acetyl-CoA carboxylase (ACC) catalyzes the first committed and regulated step in the biosynthesis of long-chain fatty acids in all living organisms(1). The bacterial form of the enzyme is composed of three different protein domains: biotin carboxylase (BC), carboxyltransferase (CT), and the biotin carboxyl carrier protein (BCCP) (2). The reaction catalyzed by ACC is shown in Scheme 1:



Scheme 1: Overall reaction catalyzed by acetyl CoA carboxylase.

Biotin carboxylase, the first domain of ACC, is responsible for catalyzing the first half-reaction, in which biotin is phosphorylated with ATP to form a carboxyphosphate intermediate. The vitamin biotin, which is covalently attached to the second domain called the biotin carboxyl carrier domain (BCCP), then accepts the carboxyl group. The third domain, carboxyltransferase, catalyzes the second half-reaction, which involves the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to produce malonyl-CoA. Both the carboxyltransferase and biotin carboxylase components retain their enzymatic activity in the absence of the other two components, and will bind free biotin as substrate without needing the biotin carboxyl carrier domain.

Each of these three proteins in acetyl-CoA carboxylase is *required* for fatty acid synthesis, which is an essential metabolic process in all animals, plants, and bacteria. This makes ACC and each of its components an effective target for antibiotics, herbicides, and even anti-obesity drugs. Strains of antibiotic resistant bacteria are becoming more and more prevalent, creating a demand for newer, more effective antibiotics. Several current antibiotics target other enzymes involved in fatty acid metabolism. In recent years, several major pharmaceutical companies have shown a keen interest in ACC as a possible target for new antibiotics.

In past years, most of the research performed on ACC has centered on the biotin carboxylase component because the gene coding for this protein has been successfully cloned and overexpressed(3). In addition, the three dimensional structure of the biotin carboxylase protein has been previously solved using x-ray crystallography(4). Little work has been done on the carboxyltransferase component in the past, although an overexpression system has recently been developed(5). This has allowed for the production of large amounts of carboxyltransferase that can be used in both structural and functional studies. Carboxyltransferase is a tetramer ($\alpha_2\beta_2$) containing two 36 kD α subunits and two 33 kD β subunits. Recently, the crystal structure for carboxyltransferase in *Staphylococcus aureus* was solved at a resolution of 2.0 Å. The *Escherichia coli* version of carboxyltransferase was also solved to a resolution of 3.2 Å. The RMSD value between the two structures was only 1.0 Å, which means the structures were nearly identical. Analysis of the protein structure revealed several active site residues as well as many other important residues that reside in the interface between subunits. Also, a distinct zinc domain in the β subunit was identified that has since been shown to bind DNA.

The crystal structure along with sequence alignments provided a basis for performing site-directed mutagenesis in order to determine the function and importance of particular residues in CT. This thesis will focus on two residues, a lysine at position 142 and an arginine at 145 on the alpha subunit. This thesis also involves a preliminary investigation of the inhibition of CT by the antibiotic tetracycline.

Materials and Methods

Reagents

Primers used were synthesized by MWG Biotech in North Carolina. The plasmid pGEM11 was purchased from Promega in Madison, WI. All restriction enzymes, dNTPs, Deep vent polymerase, and T4 DNA ligase were purchased from New England Biolabs in Cambridge, MA. The expression plasmid pET28b and the *E. coli* strain BL21DE3pLysS was purchased from Novagen in Madison, WI. Protein purification columns were HIS-Select cartridges purchased from Sigma in St. Louis, MO. Protein centrifugation filters were purchased from Amicon Centriprep in Bedford, MA. All other enzymes or reagents were from Sigma or Aldrich.

Site-Directed Mutagenesis

Site-directed mutants of carboxyltransferase were made by the PCR method of overlap extension. The template was pCZB2 and the primers are shown below in Table 1.

Table 1: Primers for Site-Directed Mutatagenesis

Mutant	Primer Sequence*
K142A	5' CCA GAA GGT TAC CGC **GCA** GCA CTG CGT CTG ATG 3' 3' GGT CTT CCA ATG GCG **CGT** CGT GAC GCA GAC TAC 5'
R145A	5' TAC CGC AAA GCA CTG **GCT** CTG ATG CAA ATG 3' 3' ATG GCG TTT CGT GAC **CGA** GAC TAC GTT TAC 5'

*The underlined bases represent the nucleotides that were changed.

The PCR products were cut with EcoR1/NdeI and XhoI, and ligated into pGEM11, forming two new plasmids. The new plasmid pBKB1 contained the mutation lysine to alanine at position 142. The other plasmid pBKB2 contained the mutation of arginine to

alanine at position 145. The wildtype gene for the β subunit was cut from pCZB1 using XhoI and BamHI and inserted in pBKB1 and pBKB2 next to the mutated α subunit genes, yielding pBKB3 and pBKB4 respectively. The insert containing the α mutant genes and the wildtype β gene was cut out of the plasmid with Nde1 and BamH1, and ligated into the expression plasmid pET28 for overexpression of the mutant enzymes. The pET28 was then transformed into *E. coli* strain BL21DE3pLysS.

Growth and Purification of Carboxyltransferase

For inoculation, 10 mL overnight cultures that contained 100 mg/mL ampicillin for the wildtype carboxyltransferase and 30 mg/mL kanamycin for the mutants were used, and bacteria were grown at 37 °C in 1 L of LB medium in 2 L flasks. The 1L samples were grown at midlog phase (approximately 3 hours). The overexpression of the carboxyltransferase gene was induced with 1g of lactose and incubated for another 2 hours. The bacterial cells were centrifuged at 10,000rpm for 10 min. at 4 °C. The harvested cells were then suspended in the following buffer—50mM NaH_2PO_4, 0.3M NaCl, 10mM Imidizole, pH 8.0—and lysed by three cycles of freezing and thawing. DNase was added to degrade the nucleic acids. The lysate was centrifuged at 20,000rpm for 1 hr. at 4 °C. Triton X-100 was added to the lysate to a concentration of 0.1% to minimize non-specific protein binding to the nickel affinity column. The lysate was loaded on a HIS-Select nickel affinity column and then extensively washed with equilibration buffer. The carboxyltransferase was eluted from the column using elution buffer (50mM NaH_2PO_4, 0.3M NaCl, 250mM Imidizole, pH 8.0). The eluted protein was dialyzed overnight against the following buffer—0.67mM EDTA, 10mM $KHPO_4$, 7.5mM KCl, pH 7.0—followed by dialysis in another buffer—500mM KCl, 10mM

HEPES, pH 7.0. CT was then concentrated using a centrifugation filter. The purified and concentrated protein was run on a 10% poly-acrylamide gel (Figure 1).

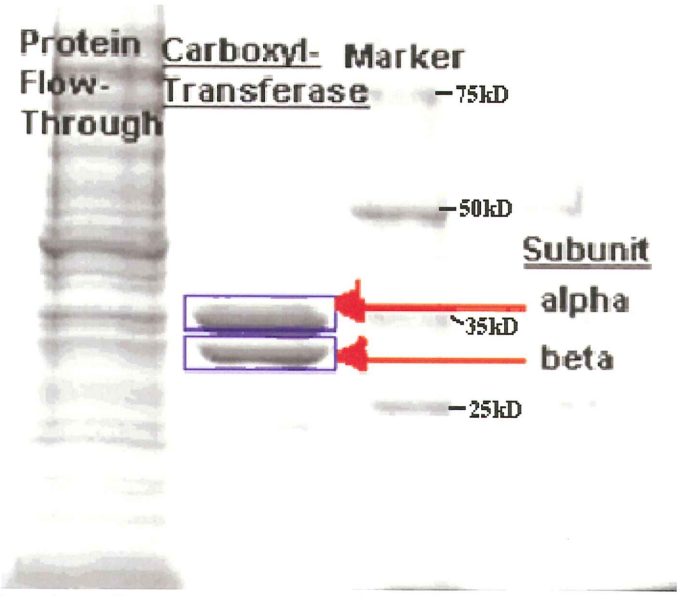
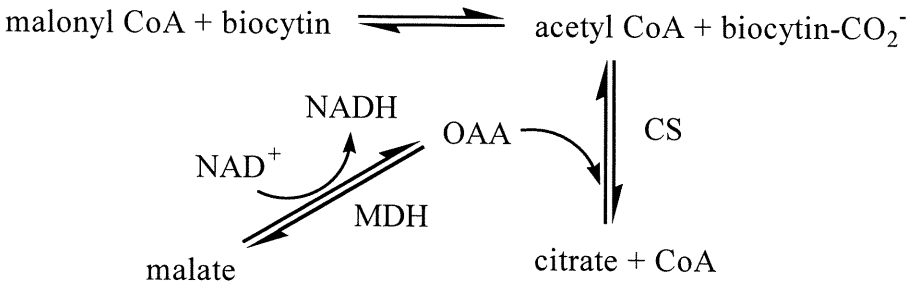


Figure 1: Purified carboxyltransferase run on a 10% poly-acrylamide gel.

The concentration of carboxytransferase was determined using the Bradford method assay using bovine serum albumin as a standard.

Steady State Kinetics

In order to determine the catalytic activity and substrate binding affinity of the mutants, a previously developed activity assay specific for carboxyltransferase was used. The activity of carboxyltransferase was assayed in the reverse direction, in which biotin and malonyl CoA served as substrates, and acetyl-CoA was produced. The production of acetyl-CoA was detected using the coupling enzymes malate dehydrogenase and citrate synthase, which reduced NAD^+ to NADH. NADH was measured spectrophotometrically at a wavelength of 340 nm(6). This enzyme coupled assay is diagrammed in Scheme 2:



Scheme 2: Enzyme coupled activity assay of carboxyltransferase. CS=citrate synthase, MDH=malate dehydrogenase, OAA=oxaloacetate

The assay reaction mixture (0.5mL) contained 100mM Tris-Cl pH 8.0, 0.5 mM NAD^+, 10 mM *L*-Malate, 0.6 mg/mL bovine serum albumin, 3.6 units/mL malic dehydrogenase, and 6.8 units/mL citrate synthase.

The K_m and V_{max} were determined by using a non-linear regression analysis where the initial velocity versus substrate concentration was fitted to the Michealis-Menten Equation. Data for competitive inhibition were fitted to Equation 1.

Equation 1: $$v = VA / (K_m(1 + I/K_{is}) + A)$$

V is the maximum velocity, A is the substrate concentration, K_m is the Michaelis constant, and K_{is} is the slope inhibition constant.

Results

Site Directed Mutagenesis

In order to locate amino acids important to the function of carboxyltransferase, a Blast search—an amino acid sequence alignment of carboxyltransferase in a wide range of bacterial species—was used to identify strictly conserved residues. On the α subunit, two conserved residues were found—a lysine at position 142 and an arginine at 145, as shown in Figure 2.

Organisms					142			145				
Escherichia Coli	P	E	G	Y	R	**K**	A	L	**R**	L	M	Q	M	A
Hemophilus influenzae	P	E	G	Y	R	**K**	A	L	**R**	L	M	E	M	A
Pasueurella multocida	P	E	G	Y	R	**K**	A	L	**R**	L	M	Q	M	A
Salmonella typhimurium	P	E	G	Y	R	**K**	A	L	**R**	L	M	E	M	A
Vibrio cholerae	P	E	G	Y	R	**K**	A	L	**R**	L	M	E	M	A
Yersinia pestis	P	E	G	Y	R	**K**	A	L	**R**	L	M	E	M	A
Caulobacter crescentus	-	-	T	V	K	**K**	H	L	**R**	A	Q	E	I	-
Mycobacterium tuberculosis	-	-	T	V	K	**K**	H	L	**R**	A	Q	E	V	-
Pseudomonas aeruguiosa	-	-	T	V	K	**K**	H	L	**R**	A	Q	A	I	-
Ralstonia solamacorium	-	-	T	V	K	**K**	H	L	**R**	A	Q	E	I	-
Mesorhizobeium loti	-	-	T	V	K	**K**	H	L	**R**	A	Q	E	I	-
Myxococcos xanthus	I	D	A	S	I	**K**	A	A	**R**	F	V	R	F	-
Veillonella parrila	I	N	A	S	D	**K**	S	S	**R**	F	I	R	F	-
Archaloglobus fulgidus	V	D	S	S	D	**K**	I	A	**R**	F	V	R	F	-
Streptomyces coelicolor	I	E	A	S	E	**K**	A	A	**R**	F	V	Q	M	-
Bacillus subtilis	I	D	A	A	D	**K**	A	A	**R**	F	I	R	F	-
Sulfolobus metallicus	I	D	A	A	D	**K**	A	A	**R**	F	I	R	F	-
Rhodococcus erythropolis	S	E	S	A	E	**K**	A	A	**R**	F	V	R	L	-

Figure 2: Sequence alignment of amino acids surrounding residues 142 and 145 on the alpha subunit of carboxyltransferase

These two amino acids were targeted for site-directed mutagenesis and were both mutated individually to alanine. For each of the mutants, the V_{max} and K_m values for malonyl CoA and biocytin were determined and compared to that of the wildtype carboxyltransferase(5).

The V_{max} for both the mutant enzymes was found to be less than that of the wildtype. Compared to wildtype, the K_m values with respect to biocytin and malonyl

CoA did not change significantly for either mutant. These results are summarized in Table 2.

Enzyme	$Km_{malonyl\ CoA}$ (μM)	$Km_{biocytin}$ (mM)	Vmax (μmol/min*mg)
Wildtype(5)	100.0±10.0	8.35±0.03	22.40±0.03
K142A	61.6±5.9	10.82±1.38	5.10±0.32
R145A	82.9±9.0	7.72±1.29	0.79±0.04

Table 2: These kinetic Parameters for wildtype carboxyltransferase and mutants K142A and R145A were determined by varying malonyl CoA at a fixed concentration of biocytin (10 mM), and by varying biocytin at a fixed concentration of malonyl CoA (100 μM). The standard errors on V_{max} and K_m were determined from the nonlinear regression analysis.

Tetracycline Inhibition

Pfizer Inc., who has shown interest in carboxyltransferase as a target for antibiotics, performed a high throughput screen that revealed tetracycline, a common antibiotic, was an inhibitor of carboxytransferase. More extensive inhibition studies were done to determine the mechanism of inhibition with respect to each substrate and the magnitude of inhibition. The inhibition study with respect to malonyl CoA was performed by varying malonyl CoA at fixed levels of biocytin with and without tetracycline. The tetracycline used in this study was not water soluble, so the tetracycline was instead dissolved in ethanol. The highest concentration of tetracycline that could be used for the inhibition study was 0.15 mM. Inhibition by tetracycline with respect to malonyl CoA exhibited competitive inhibition as shown in Figure 3.

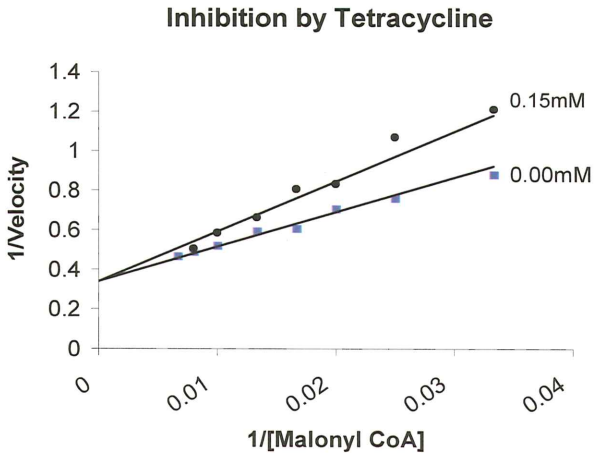


Figure 3: Double-reciprocal (Lineweaver-Burk) plot of inhibition of CT by tetracycline while varying malonyl CoA at a fixed biocytin concentration (10 mM). The points shown are the reciprocal of the experimental velocities and the lines are derived from the best fit of the data to Equation 1 in Materials and Methods.

When this data was fit to Equation 1 from Materials and Methods, the K_{is} was determined to be 0.34±0.06 mM. The inhibition type with respect to bioctyin could not be determined due to the fact that tetracycline levels could not increased above its solubility limit of 0.15 mM.

Discussion

Site Directed Mutagenesis

Based on a sequence alignment, the lysine residue at postion 142 and the arginine residue at 145 were found to be strictly conserved. Strictly conserved residues are presumably important to the function of the enzyme, so to test their importance, these amino acids were mutated by site-directed mutagenesis. In addition to being sequentially close to each other, the mutated residues both had similar kinetic data, which suggests that the two amino acids have common functions. The K142A mutant was found to have very similar K_m values for both biocytin and malonyl CoA when compared to wildtype. However, the maximal activity of K142A was 22.7% of the wildtype maximal velocity. Likewise, the K_m values of K145A with respect to biocytin and malonyl CoA were nearly identical to that of wildtype, while the maximal activity of K145A was 3.5% of the wildtype value. These decreases in maximal activity are significant, but not characteristic of active site residues. Most likely these amino acids serve some other structural purpose within carboxyltransferase.

Crystals of carboxyltransferase from E. coli were grown and diffracted to a resolution of 3.0 angstroms. From this data, the structure was solved and has provided additional information on the location and probable function of the mutated residues. As shown in Figure 4, the structure shows that carboxyltransferase is indeed a heterotetramer containing two α subunits and two β subunits.

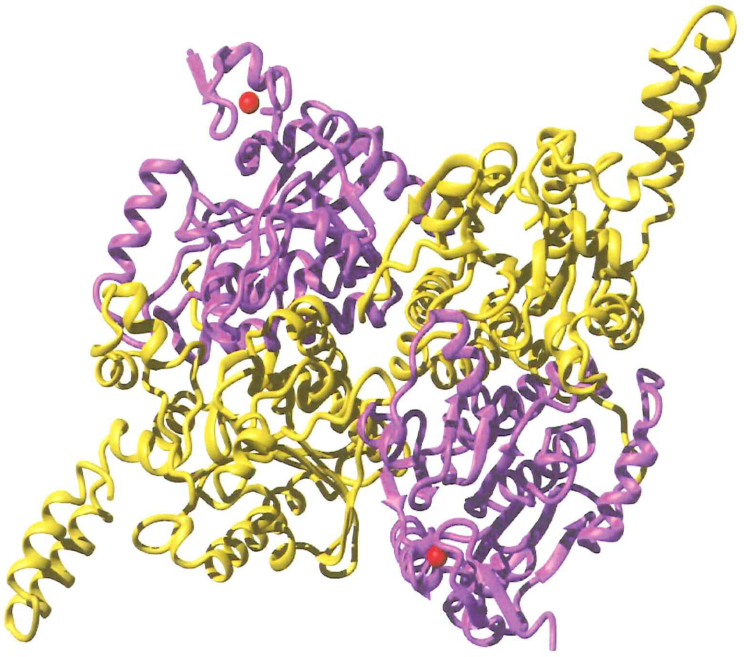


Figure 4: Structure of carboxyltransferase; α subunits shown in yellow, β subunits shown in purple

One α subunit and one β subunit are joined to form a catalytic dimer that contains a large active site pocket spanning both subunits, seen below in Figure 5.

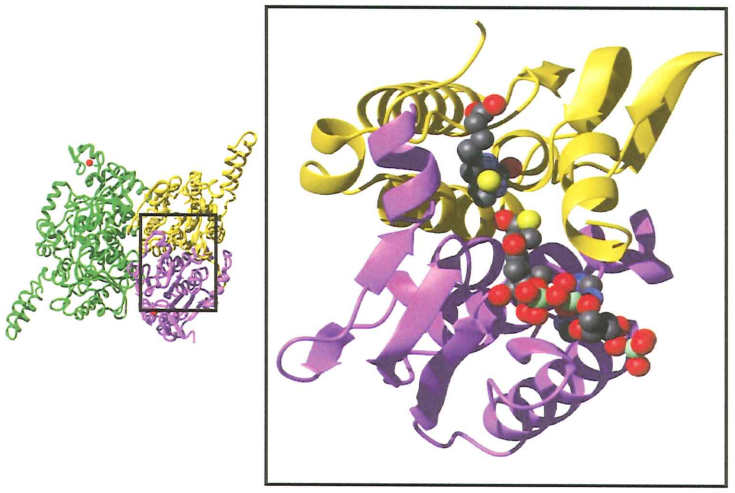


Figure 5: The α subunit (yellow) and β subunit (purple) join to form a catalytic dimer that contains the active site (shown binding biotin and malonyl CoA in blow up at right). One of the two catalytic α β dimers is shown in green on the left.

The interface between the α and β subunits within the same catalytic dimer is essential for maintaining the structure of the active site. Two catalytic α β dimers are joined together

at the weak interface to form the complete heterotetramer. According to the structure, both position 142 and 145 on the α subunit can be found at this interface between the two catalytic dimers. As shown in Figure 6, residues 142 and 145 are located on the α subunit near the interface with the β subunit on the opposite catalytic dimer.

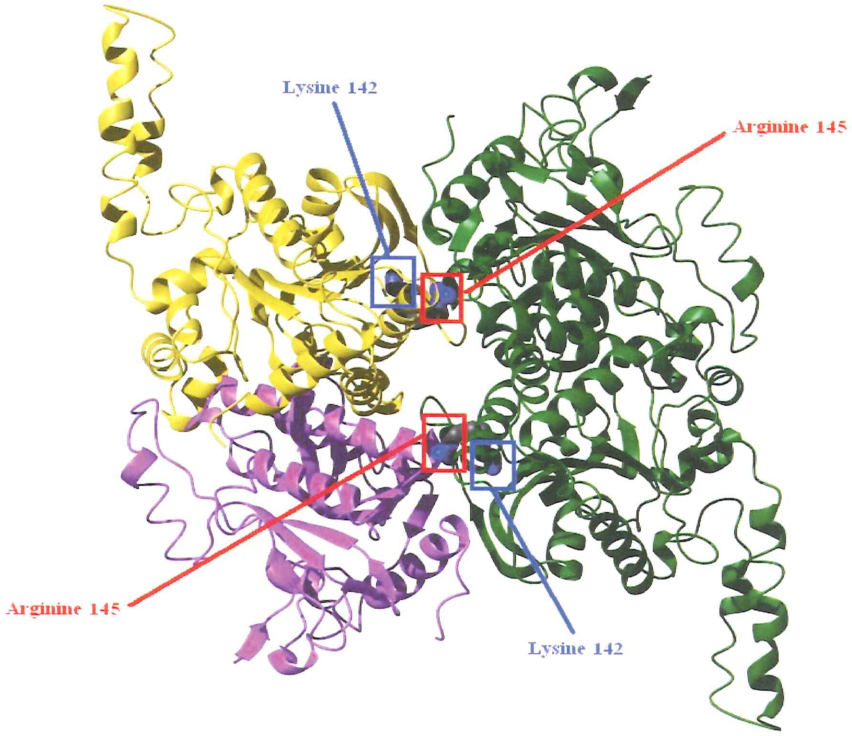


Figure 6: Positions of Lysine 142 (in blue) and Arginine 145 (in red) on carboxyltransferase. The α subunit is shown in yellow, the β subunit is shown in purple, and an α β catalytic dimer is shown in green.

Lysine 142 does not interact directly with β subunit of the other dimer. However, the lysine does appear to interact with a loop on the same α subunit that forms a salt bridge with the β subunit of the opposite dimer. As shown in Figure 7, the positive lysine is surrounded by several negative aspartates. It is possible that the lysine interacts with these aspartates and stabilizes the loop on the α subunit, promoting the interaction between the α and β subunits at the weak interface.

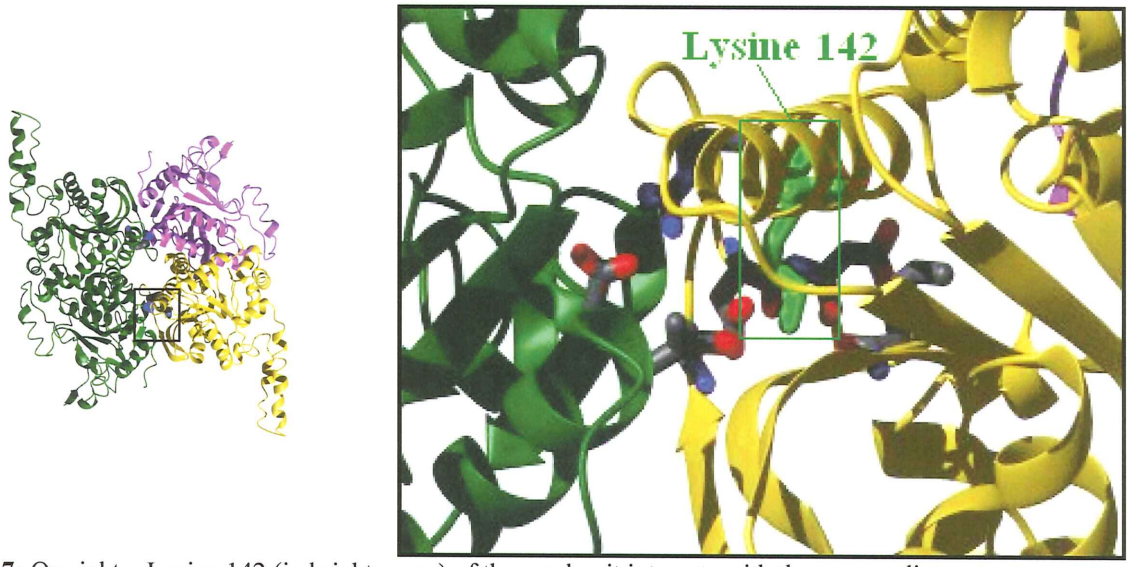


Figure 7: On right—Lysine 142 (in bright green) of the α subunit interacts with the surrounding oxygens (red molecules) of several aspartates on the same α subunit. The α subunit is shown in yellow, the β subunit is shown in purple, and an α β catalytic dimer is shown in green.

The kinetic data of K142A showed that mutating the lysine does not change the K_m values of the enzyme and only slightly lowers the maximum activity of the enzyme. This suggests that the mutated amino acid is not critical to the function of the enzyme, but nonetheless the decrease in maximal activity shows that the residue still has importance to the enzyme. This is congruent with the suggestion that the lysine merely stabilizes a loop at the weak interface which promotes interaction between the subunits. Even though the lysine at 142 was shown to be strictly conserved, its function does not appear to be critically important.

The arginine at 145 does appear to directly interact with the β subunit at the weak interface. The arginine is about 3 angstroms from an aspartate at position 255 of the β subunit, as shown in Figure 8. Due to proximity and the opposite charges between the two residues, it is highly probable that there is a salt bridge between them.

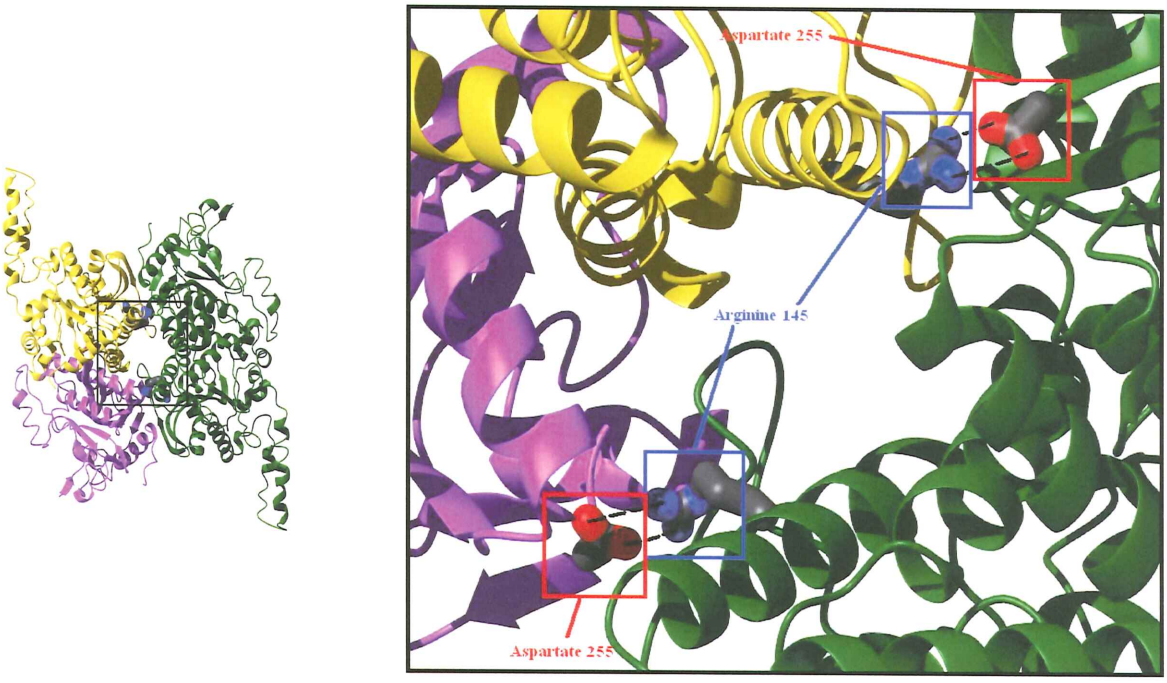


Figure 8: On right—Arginine 145 (in blue) of the α subunit shown interacting with Aspartate 255 (in red) of the β subunit at the weak interface. The α subunit is shown in yellow, the β subunit is shown in purple, and an α β catalytic dimer is shown in green.

It is very likely that this arginine directly stabilizes the interaction between the α and β subunits at the weak interface. The kinetic data of R145A showed that mutating arginine did not change the K_m values of the enzyme but it did decrease the maximal activity by 30-fold. This suggests that arginine 145 has a more important role than lysine 142 in the overall function of the enzyme. Due to the position of arginine 145 and its importance in maintaining the activity of the protein, it is proposed that the arginine does form a direct interaction with the β subunit, and it is likely that this interaction is important in maintaining this interface. The decrease in activity seen in the mutants may be a sign that the interaction between the two catalytic dimers was disrupted to the point of dissociation.

The idea that the interaction between the two catalytic dimers is important to the function of the overall enzyme has unique implications. Since each catalytic dimer has its own active site, it would be expected that each dimer could function independently.

Although this notion has not been disproved, the two mutations discussed in this paper did alter the performance of the enzyme, and did so by interfering with the interface between the two catalytic dimers. This suggests that each catalytic dimer performs optimally only when it is fully interacting with another catalytic dimer. This phenomenon was also observed in biotin carboxylase(7), although a full explanation for this behavior has not been established.

Tetracycline Inhibition

Tetracycline is a common broad-spectrum antibiotic used in both clinical and laboratory settings. The structure of tetracycline is shown below in Figure 9.

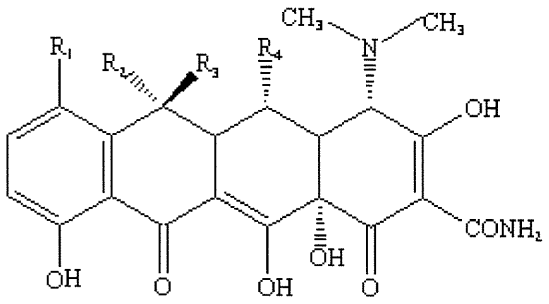


Figure 9: Structure of tetracycline

Tetracycline is known to bind to and inhibit translation in the ribosome(8). Recently, tetracycline was shown to inhibit carboxyltransferase in a high throughput screen performed by Pfizer Inc. The work in this thesis showed that tetracycline exhibited competitive inhibition versus malonyl CoA, as shown in Figure 3 from results. The K_{is} was found to be 0.34±0.06 mM. The type of inhibition with respect to biocytin could not be determined because tetracycline was not water soluble and could not be tested at levels above 0.15 mM. To overcome this problem, tetracycline will be dissolved in DMSO in future inhibition studies involving carboxyltransferase. Although the mechanism of

inhibition for CT by tetracycline is poorly defined and needs further experimentation, the discovery that tetracycline inhibits CT at all is of importance. Bacteria are very adept at mutating and gaining antibiotic resistance, so a mutation in the ribosome of the bacteria might prevent tetracycline from binding to and inhibiting transcription. However, even if this did occur, tetracycline would still be effective because it can also inhibit carboxyltransferase. This would interfere with the fatty acid metabolism of the bacterial cell, another essential metabolic process.

Tetracycline showed competitive inhibition versus malonyl CoA, which suggests that tetracycline perhaps binds to the active site of carboxyltransferase. However, the structure of tetracycline (Figure 9) does not resemble either of the substrates of CT, so the binding of tetracycline to the active site seems unlikely. Another possible explanation for the binding site of tetracycline was uncovered with the discovery of the CT structure. The solved structure of carboxyltransferase revealed the presence of a zinc finger domain on the β subunit, seen in Figure 10. This led to the discovery that carboxyltransferase is a DNA binding protein.

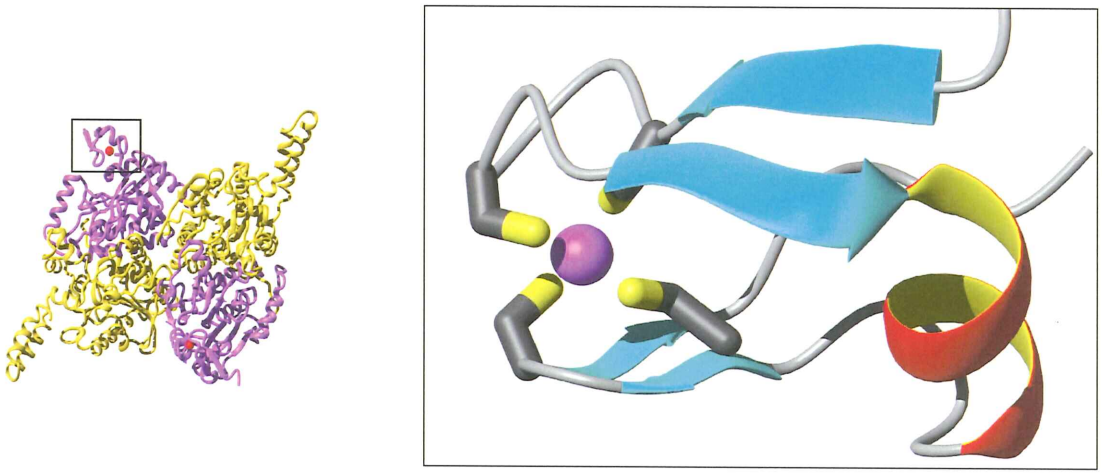


Figure 10: Zinc finger domain on β subunit of carboxyltransferase. On Right—four Cysteine residues (yellow molecules) bind a zinc atom (purple). On Left—the α subunit is shown in yellow and the β subunit is shown in purple.

A past study of carboxyltransferase in the chromosome of plants in which the cysteine residues of the zinc domain were mutated, showed that this domain was in fact required for catalytic activity of the enzyme(9). Therefore, it is possible that tetracycline binds to and inhibits this zinc domain, thereby inhibiting the overall activity of the enzyme. Further inhibition studies involving tetracycline and DNA binding would have to be performed in order to validate this possibility.

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