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The role of cysteine 230 and lysine 238 of biotin carboxylase in the deprotonation of biotin and synthesis of bisubstrate analogy inhibitor of carboxyltransferase

Keith Logan Levert
Louisiana State University and Agricultural and Mechanical College

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THE ROLE OF CYSTEINE 230 AND LYSINE 238 OF BIOTIN CARBOXYLASE IN THE DEPROTONATION OF BIOTIN AND SYNTHESIS OF A BISUBSTRATE ANALOG INHIBITOR OF CARBOXYLTRANSFERASE

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
Keith Logan Levert
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ABSTRACT

Acetyl-CoA carboxylase catalyzes the first step in the synthesis of fatty acids. The *Escherichia coli* form of the enzyme consists of a biotin carboxylase protein, a biotin carboxyl carrier protein, and a carboxyltransferase protein. This enzyme uses the cofactor biotin as a carboxyl carrier. In order for the carboxylation of biotin to occur, biotin must be deprotonated at its N-1 position. It has been proposed that the active site residues cysteine 230 and lysine 238 act as an acid-base pair to deprotonate biotin. To test this hypothesis, site-directed mutagenesis was used to mutate cysteine 230 to alanine (C230A) and lysine 238 to glutamine (K238Q). Mutations at either residue resulted in a 50-fold increase in the $K_m$ for ATP. The C230A mutation had no effect on the formation of carboxybiotin, indicating that cysteine 230 does not play a role in the deprotonation of biotin. However, the K238Q mutation resulted in no formation of carboxybiotin, which showed that lysine 238 has a role in the carboxylation reaction. However, the pK value for lysine 238 was 9.4 or higher, suggesting lysine 238 is not a catalytic base. Thus, the results suggest that cysteine 230 and lysine 238 do not act as an acid-base pair in the deprotonation of biotin.

A bisubstrate analog inhibitor of carboxyltransferase was synthesized by covalently linking biotin to Coenzyme A via an acyl bridge between the sulfur of Coenzyme A and the N-1 of biotin. The inhibitor was found to have an inhibition constant of $23 \pm 2 \mu$M, which means it binds the enzyme 350-times tighter than biotin. The bisubstrate analog demonstrated competitive inhibition versus malonyl-CoA and
noncompetitive inhibition versus biocytin. This is consistent with an ordered kinetic mechanism with malonyl-CoA binding first. A precursor to the inhibitor, chloroacylated biotin, was capable of inhibiting the differentiation of 3T3-L1 cells in a dose-dependent manner. Treatment with chloroacylated biotin resulted in a decrease in acetyl-CoA carboxylase activity and inhibited lipid accumulation. Our results support recent studies that indicate acetyl-CoA carboxylase may be a suitable target as an anti-obesity therapeutic.
Biotin

Biotin was discovered in 1936 when it was isolated from egg yolk and shown to be a growth factor for yeast (Kögl and Tönnis, 1936). The first evidence suggesting that biotin was involved in carbon dioxide metabolism was obtained in 1949, when the incorporation of $^{14}$CO$_2$ into aspartate by *Lactobacillus arabinosum* was shown to be dependent on the presence of biotin in the growth medium (Lardy *et al*., 1949). The dependence of biotin on carboxyl transfer reactions was further supported when biotin-deficient rats were found to fix lower levels of $^{14}$CO$_2$ into cellular metabolites, such as aspartate and citrate, than did normal rats (MacLeod and Lardy, 1949). In 1950, biotin again was found to be a necessary component of carboxylation reactions, the incorporation of $^{13}$CO$_2$ into oxaloacetate in cell-free extracts of *Micrococcus lysodeikticus* also was found to be dependent on the presence of biotin (Wessman and Werkman, 1950). However, it was not until 1958 that a direct association between biotin and an enzyme was first discovered (Wakil *et al*., 1958). This association was discovered when purified avian liver acetyl-CoA carboxylase was found to contain biotin, providing the first evidence that biotin is a cofactor for enzymes. It has since been determined that biotin is a cofactor for numerous enzymes that are involved in carboxylation, decarboxylation, and carboxyl transfer reactions (Moss and Lane, 1971).
Structure and Chemistry of Biotin. The structure of biotin was first determined through chemical analysis of purified biotin (Melville et al., 1942), and later confirmed through its chemical synthesis (Harris et al., 1944). Biotin consists of two five-membered rings (ureido and tetrahydrothiophene rings) fused cis to one another (Figure 1.1). Extending from the tetrahydrothiophene ring is a valeric acid side chain. The valeric acid side chain also has a cis configuration, with respect to the ureido ring. Biotin contains three chiral carbon atoms, which can lead to eight total stereoisomers. However, only the isomer described above is biologically active.

![Figure 1.1](structure_of_biotin.png)

**Figure 1.1** Structure of biotin.

The requirement of biotin in carboxylation reactions suggests biotin acts as a carboxyl group carrier. Evidence for this role of biotin was first demonstrated when a methyl ester derivative of carboxybiotin was isolated from the reaction catalyzed by β-methylcrotonyl-CoA carboxylase, in which biotin was carboxylated at the 1’-N atom (Lynen et al., 1961). Further evidence for this role of biotin was obtained when Lane’s group demonstrated that chemically synthesized carboxybiotin, carboxylated at the 1’-N
position, could be used as a substrate for acetyl-CoA carboxylase (Guchhait et al., 1974b). Since biotin is carboxylated at the 1’-N position the reactive form of biotin is probably not the form shown in Figure 1.1. The 1’-N atom for the tautomer of biotin shown in Figure 1.1 is a weak nucleophile due to the amide resonance of the ureido ring. Deprotonation of the 1’-N atom would result in the tautomerisation of biotin into an enol-like form, increasing the nucleophilicity of this atom (Hegarty et al., 1969). This suggests the possibility of an active-site catalytic base that can deprotonate biotin prior to carboxylation (Scheme 1.1).

![Scheme 1.1](image)

X-ray crystallographic studies of biotin support the notion that enolization is important for the chemistry of this molecule. Stallings and DeTitta (1985) performed high-resolution crystallographic studies on biotin and biotin derivatives. They found that the carbonyl bond on the ureido ring was considerably longer than the previously accepted value. Additionally, the C2’-N1’ and C2’-N3’ bonds were both shorter than the accepted value. This results in a polarized ureido ring that can interact with ions and polar compounds. Stallings and DeTitta argue that the polarized nature of the ureido ring predisposes biotin to activation by enolization.
Nutritional Aspects of Biotin. Biotin, or vitamin H, cannot be synthesized by animals as part of their normal metabolism, and is therefore an essential nutrient. Plants, most bacteria, and some fungi are capable of synthesizing biotin, and biotin is found in many food sources. In addition, biotin is synthesized in sufficient quantities by human intestinal bacteria to satisfy our daily requirements for this vitamin. Therefore, disease states related to biotin deficiency are extremely rare. However, a biotin deficient state can occur if an individual’s diet consists of foods that are high in avidin. Avidin is a protein that binds biotin with an association constant of $10^{15} \text{ M}^{-1}$, which is the tightest association between a protein and ligand found in nature. This tight binding of biotin by avidin will prevent biotin’s absorption into the bloodstream. Biotin deficiency is also associated with prolonged parenteral nutrition, severe malnutrition, and inherited metabolic disorders (Melendez, 2000). Autosomal recessive disorders in humans that result in a biotin deficient state have been traced to mutations in the genes coding for biotinidase and biotin ligase, two enzymes involved in biotin metabolism. The major symptom of biotin deficiency is a severe dermatitis. Biotin deficiency has also been shown to lead to impaired glucose tolerance (Bender, 1999), and biotin deficiency is teratogenic in several mammalian species (Mock et al., 2002).

Biotin-Dependent Enzymes

Biotin, or vitamin H, is a cofactor for a diverse group of enzymes that play a role in many metabolic pathways. The role of biotin is to act as a carboxyl carrier, transferring a carboxyl group from a donor to an acceptor molecule, and biotin-dependent
enzymes can catalyze carboxylation, decarboxylation, and carboxyl transfer reactions, as shown below:

\[
\begin{align*}
\text{HCO}_3^- + \text{ATP} + \text{RH} & \quad \overset{\text{Mg}^{2+}}{\longrightarrow} \quad \text{ADP} + \text{P}_1 + \text{RCO}_2^- + \text{H}^+ \quad \text{(carboxylation)} \\
\text{RCO}_2^- + \text{R'H} & \quad \overset{\text{ATP}}{\longrightarrow} \quad \text{RH} + \text{R'CO}_2^- \quad \text{(carboxyl transfer)} \\
\text{RCO}_2^- + \text{H}_2\text{O} + 2 \text{Na}^+_{\text{intracellular}} & \quad \overset{\text{ADP}}{\longrightarrow} \quad \text{RH} + \text{HCO}_3^- + 2 \text{Na}^+_{\text{extracellular}} \quad \text{(decarboxylation)}
\end{align*}
\]

The most common and widespread biotin-dependent enzymes are those that catalyze carboxylation reactions. In fact, all known eukaryotic biotin-dependent enzymes fall into this category. Those enzymes that catalyze carboxylation reactions in mammals and the metabolic process they are involved in are pyruvate carboxylase in gluconeogenesis, acetyl-CoA carboxylase in fatty acid synthesis, propionyl-CoA carboxylase in fatty acid oxidation, and β-methylcrotonyl-CoA carboxylase in amino acid catabolism. There are also two biotin-dependent carboxylases that are found only in microorganisms. Bacteria that can use urea as a nitrogen source utilize the enzyme urea carboxylase, and geranyl-CoA carboxylase is found in microbes that can catabolize isoprenoid compounds. The biotin-dependent decarboxylases are less numerous and are only found in anaerobic prokaryotes. These enzymes are used to pump sodium out of the cell without the use of ATP. There are three known biotin-dependent decarboxylases, which are named according to the molecule that donates the carboxyl group, oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, and glutaconyl-CoA decarboxylase. The enzyme transcarboxylase is the only representative of the biotin-dependent transcarboxylases.
This enzyme is found in the bacterium *Propionibacterium shermanii*, where it is used for the formation of propionic acid.

The overall reactions catalyzed by biotin-dependent enzymes have been shown to follow ping-pong kinetics (Wood and Barden, 1977). The exchange reactions characteristic with this mechanism, along with the isolation of the carboxybiotin intermediate, suggest that the reactions catalyzed by biotin-dependent enzymes can be broken down into two discrete steps, each catalyzed at a separate active-site. In this mechanism biotin acts as a carboxyl carrier, transferring the carboxyl group between the two active sites of the enzyme. The two half reactions catalyzed by biotin-dependent enzymes are as follows: (1) The transfer of the carboxyl group from the carboxyl donor to biotin, forming the carboxybiotin intermediate and (2) the transfer of the carboxyl group from carboxybiotin to the carboxyl acceptor. This enzyme mechanism was confirmed when *Escherichia coli* acetyl-CoA carboxylase was isolated and found to consist of three distinct subunits, two of which catalyze only one half of the overall reaction (Guchhait *et al.*, 1974a). The third subunit of the enzyme complex contains the biotin cofactor bound to a specific lysine residue, thus allowing the biotin cofactor to move between the two active sites of the enzyme.

This third subunit, or biotinyl domain, is common to all biotin-dependent enzymes. The biotinyl domain contains biotin covalently linked to the enzyme via the ε-amino group of a specific lysine residue and the carbonyl of the valeric acid side chain of biotin (Figure 1.2). Thus, all biotin-dependent enzymes must undergo a posttranslational
modification. This is accomplished by the enzyme biotin ligase, which covalently attaches biotin to the enzyme (Chapman-Smith and Cronan, 1999). Once biotin is linked to the enzyme it can act as a shuttle, transferring the carboxyl group between the two active sites of the enzyme (Guchhait et al., 1974a).

**Biotin-Dependent Carboxylases**

The primary focus of this dissertation will be the carboxylation reactions of biotin-dependent enzymes. Biotin-dependent carboxylases play vital roles in many metabolic pathways, including gluconeogenesis, fatty acid synthesis, and amino acid catabolism. All biotin-dependent enzymes that catalyze carboxylation reactions utilize bicarbonate as the source for CO₂ and proceed through the following two-step pathway:
In the first half of the reaction biotin is carboxylated in an ATP dependent reaction, producing the carboxybiotin intermediate where biotin is carboxylated at the 1′-N position. The role of ATP in this reaction is to activate bicarbonate, forming the reactive acyl phosphate intermediate carboxyphosphate. In the second half of the reaction the carboxyl group is transferred from carboxybiotin to the acceptor molecule. The acceptor molecule will vary depending on the enzyme. For example, pyruvate carboxylase will utilize pyruvate as the carboxyl acceptor and acetyl-CoA carboxylase utilizes acetyl-CoA as the acceptor molecule. As for all biotin-dependent reactions, biotin remains covalently linked to the enzyme throughout the reaction.

**Acetyl-CoA Carboxylase**

The characteristics of the reactions catalyzed by biotin-dependent carboxylases (the ping-pong kinetics with each half reaction being catalyzed at a distinct active site, and the fact that biotin remains covalently linked to the enzyme throughout the reaction) have presented enzymologists a challenging task in resolving the catalytic mechanism of these enzymes. Fortunately, the acetyl-CoA carboxylase enzyme complex from *Escherichia coli* has provided researchers with an ideal system to study biotin-dependent reactions (*Guchhait et al.*, 1974a). The three domains of this biotin-dependent enzyme
are each contained on separate protein subunits. Two of these subunits, biotin carboxylase and carboxyltransferase, represent the catalytic domains. Biotin carboxylase is responsible for catalyzing the first half of the reaction and carboxyltransferase catalyzes the second half of the reaction. The third subunit, the biotin carboxyl carrier protein, contains biotin covalently linked to lysine 122 (Chapman-Smith and Cronan, 1999). The two catalytic subunits of the enzyme complex each retain activity when isolated from the other subunits. Both of these subunits will also recognize free biotin as a substrate, eliminating the need for the biotin carboxyl carrier protein during kinetic analysis. Acetyl-CoA carboxylase is one of only two biotin-dependent carboxylases that can recognize free biotin as a substrate, the other being β-methylcrotonyl-CoA carboxylase (Moss and Lane, 1971). Since the isolation of acetyl-CoA carboxylase from *Escherichia coli*, the genes encoding the three subunits have each been cloned and overexpressed (Li and Cronan, 1992a,b; Nenortas and Beckett, 1996; Blanchard and Waldrop, 1998). Furthermore, the three-dimensional structure of biotin carboxylase has been solved by x-ray crystallography (Waldrop *et al*., 1994; Thoden *et al*., 2000a). All of this taken together aids in simplifying the study of acetyl-CoA carboxylase, which provides insight into the catalytic mechanisms of all biotin-dependent enzymes.

**Structure of Biotin Carboxylase.** Biotin carboxylase is a homodimer consisting of two 50 kilodalton polypeptide chains. The x-ray crystal structure of this protein was originally solved to 2.4 Å resolution, and has recently been refined to 1.9 Å, representing the only known three-dimensional structure of a biotin-dependent enzyme (Waldrop *et al*., 1994; Thoden *et al*., 2000a). Each subunit of the biotin carboxylase homodimer can
be divided into three structural domains. The N-terminal domain, consisting of residues Met 1-Ile 103, is composed of five strands of parallel $\beta$-pleated sheets flanked on either side by a total of four $\alpha$-helices. Following the N-terminal domain the polypeptide chain extends into the B-domain, which consists of two $\alpha$-helices and three antiparallel $\beta$-pleated sheets. The residues that make up the B-domain include Val 131-Tyr 203. From the B-domain the polypeptide chain extends into the C-terminal domain, consisting of residues Arg 208 through the C-terminus. The main structural component of this domain is composed of an eight-stranded antiparallel $\beta$-pleated sheet. This domain also contains a three-stranded antiparallel $\beta$-pleated sheet and seven $\alpha$-helices. Overall the biotin carboxylase monomer forms a relatively compact structure, but the B-domain forms a somewhat isolated protrusion extending from the N and C-terminal domains (Figure 1.3). The region of the polypeptide between the B-domain and the main body of the protein was not well defined in the x-ray structure, suggesting a high degree of flexibility in this area. Consistent with this observation is the composition of the amino acid sequence in the polypeptide chain that connects to the B-domain. The amino acid sequence in this region is rich in glycine residues, again indicating the possibility for conformational flexibility. Waldrop et al. (1994) suggested the role of the B-domain is to act as a lid that closes down on the active site once the substrates are bound and positioned for catalysis. This hypothesis was recently confirmed when the crystal structure of biotin carboxylase was determined with ATP bound in the active site (Thoden et al., 2000a). The structure of biotin carboxylase with the nucleotide bound clearly demonstrated movement of the B-domain, which acts as a lid to cover the active site of the enzyme.
The protein is composed of three separate structural motifs, the N-, B-, and C-domains which are color-coded magenta, yellow, and red, respectively. The B-domain forms a protrusion extending from the N- and C-terminal domains, and acts like a lid to close over the active site of the enzyme after the substrates bind. The ribbon representation was prepared using the program RasMol.

**Figure 1.3** Ribbon representation of one subunit of the biotin carboxylase homodimer.
Soon after the three-dimensional structure of biotin carboxylase was determined, Artymiuk et al. (1996) reported striking similarities between the overall structure of this enzyme and two peptide ligases, glutathione synthetase and D-alanine:D-alanine ligase. The high degree of similarity in the structure of these enzymes is especially interesting considering these enzymes only share 11% primary sequence identity. However, these enzymes all catalyze very similar reactions. All these enzymes couple the hydrolysis of ATP to the formation of a carbon-nitrogen bond between a carboxyl group and an amino group, and their catalytic mechanisms include an acyl phosphate intermediate (Scheme 1.2) (Artymiuk et al., 1996; Galperin and Koonin, 1997). Soon after the similarities in these enzymes were noted the structure of carbamyl phosphate synthetase was determined (Thoden et al., 1997). The structure of carbamyl phosphate synthetase, as well as the reaction it catalyzes, also demonstrates homology to biotin carboxylase, glutathione synthetase, and D-alanine:D-alanine ligase. Since these enzymes possess strong structural and mechanistic similarities, they became the charter members of the ATP-grasp family of enzymes. The name “ATP-grasp” derives from the unusual nucleotide-binding fold found in these enzymes, where ATP binds in a cleft formed by

$$\text{R} - \text{C} = \text{O} \quad \text{ATP} \quad \text{R} - \text{C} = \text{O} \quad \text{PO}_3^{2-} \quad \text{R'} \cdot \text{NH} \cdot \text{R''} \quad \text{HOPO}_3^{2-}$$

Scheme 1.2
structural elements composed of antiparallel β-pleated sheets (Galperin and Koonin, 1997). A search of protein sequence databases using the position-specific iterative BLAST program was performed by Galperin and Koonin (1997) in an attempt to identify new members of this family of proteins. As a result of their search the biotin-dependent carboxylase domains of pyruvate carboxylase and propionyl-CoA carboxylase were added to the list of ATP-grasp enzymes. Their search also identified urea amidolyase, tubulin-tyrosine ligase, and three enzymes of purine biosynthesis as members of this superfamily of enzymes. Since that time, the three-dimensional structure of two of the enzymes involved in purine biosynthesis have been determined, \( N^5 \)-carboxyaminoimidazole transformylase (Thoden et al., 1999) and glycinamide ribonucleotide transformylase (Thoden et al., 2000b), and both of these enzymes demonstrate the predicted structural homologies. The crystal structure of glycinamide ribonucleotide synthetase, another enzyme involved in purine biosynthesis, revealed this enzyme also shares the same structural homologies, adding a new member to the ATP-grasp family of enzymes (Wang et al., 1998).

**Biotin Carboxylase Reaction Mechanism.** The biotin carboxylase component of acetyl-CoA carboxylase catalyzes the first half of the overall reaction of this enzyme complex, namely the ATP dependent carboxylation of biotin with bicarbonate serving as the source of \( \text{CO}_2 \). The role of ATP in this reaction is to activate bicarbonate by forming the intermediate compound carboxyphosphate (Scheme 1.3). Evidence for this role of ATP comes from the observation of an exchange of \(^{18}\text{O}\) between \( \text{H}^{18}\text{O}_3 \) and \( \text{P}_i \) in
reactions catalyzed by the biotin-dependent carboxylases, propionyl-CoA carboxylase and biotin carboxylase (Kaziro et al., 1962; Ogita and Knowles, 1988). This exchange of $^{18}$O suggests a direct interaction between bicarbonate and the $\gamma$-phosphate of ATP.

Further evidence supporting a carboxyphosphate intermediate comes from the finding that both the biotin carboxylase component of acetyl-CoA carboxylase as well as pyruvate carboxylase will catalyze the phosphorylation of ADP in the presence of carbamyl phosphate (Polakis et al., 1972; Ashman and Keech, 1975). This ATP synthesis reaction is thought to represent the reaction in the non-physiological direction with carbamyl phosphate acting as an analog of carboxyphosphate (Polakis et al., 1974).

Carboxyphosphate is an unstable compound with an estimated half-life of 70 msec (Sauers et al., 1975). This makes the physical detection of this proposed reaction intermediate quite difficult. However, there is precedent for the competence of this intermediate. Carboxyphosphate has been observed in the reaction catalyzed by carbamyl phosphate synthetase by both positional isotope exchange (PIX) studies and by direct trapping of the intermediate. Carbamyl phosphate synthetase catalyzes the production of carbamyl phosphate from ATP, bicarbonate, and ammonia in a reaction scheme that is virtually identical to the mechanism proposed for the production of

![Scheme 1.3](image-url)
carboxybiotin by the biotin-dependent carboxylases. PIX can be observed with enzymes in which ATP reversibly phosphorylates an acceptor molecule (bicarbonate in the case of carbamyl phosphate synthetase and the biotin-dependent enzymes). With carbamyl phosphate synthetase PIX was observed as the bridge oxygen to non-bridge oxygen scrambling that results from rotation of the $\beta$-phosphate prior to reversal of the reaction (Wimmer et al., 1979). The carboxyphosphate intermediate has also been successfully trapped by forming its more stable trimethyl ester (Powers and Meister, 1976).

It is uncertain whether carboxybiotin arises from a reaction between biotin and carboxyphosphate or whether the carboxyphosphate first dissociates to carbon dioxide. The existence of carbon dioxide as an enzyme intermediate has never directly been tested for in the reaction of biotin carboxylase, however, Gibson et al. (1998) recently tested for the existence of carbon dioxide as an intermediate in the reaction catalyzed by carbamyl phosphate synthetase. Carbamyl phosphate synthetase will catalyze a bicarbonate-dependent ATPase reaction in the absence of a nitrogen source. This reaction proceeds through the formation of carboxyphosphate, and in the absence of a nitrogen source carboxyphosphate breaks down into bicarbonate and phosphate. If carboxyphosphate were to first dissociate into carbon dioxide, then the presence of this intermediate could be detected through changes in the pH of the medium. For example, if carboxyphosphate were to decompose during the reaction through direct attack by a water molecule one proton would be liberated for every turnover of the enzyme, and the rate of ATP hydrolysis would equal the rate of proton release. However, if carboxyphosphate were to dissociate to carbon dioxide and phosphate and carbon dioxide was released, then the rate
of proton production would rely on the nonenzymatic hydration of carbon dioxide to bicarbonate. If the rate of ATP hydrolysis were made to proceed faster (by adding more enzyme) than the nonenzymatic rate of hydration, then the intermediacy of carbon dioxide would be characterized by a lag in the time course for proton production. When these rates were measured under conditions where ATP hydrolysis occurred faster than the nonenzymatic rate of carbon dioxide hydration, both the rate of ATP hydrolysis and proton production were equal. This suggests that carboxyphosphate is directly attacked by water and does not first dissociate to carbon dioxide and phosphate. Gibson et al. (1998) also monitored the reaction using NMR spectroscopy while H\textsuperscript{13}CO\textsubscript{3}\textsuperscript{−} was used as substrate and again found no evidence for carbon dioxide production. With no evidence to support the existence of carbon dioxide as an enzyme intermediate, the favored mechanism for carbamyl phosphate synthetase suggests carboxyphosphate directly reacts with the nitrogen atom of ammonia. By inference the reaction catalyzed by biotin carboxylase proceeds through a direct transfer of carbon dioxide from carboxyphosphate to the 1’-N of biotin.

Further support for the direct transfer of carbon dioxide from carboxyphosphate to biotin arises from a comparison between the reaction of biotin carboxylase and the reactions catalyzed by other members of the ATP-grasp enzymes. All reactions catalyzed by the ATP-grasp enzymes utilize ATP to activate a carboxyl group, through the formation of an acyl phosphate intermediate (Artymiuk et al., 1996; Galperin and Koonin, 1997). Given the high degree of similarity between these enzymes, both structurally and mechanistically, it is tempting to consider an equivalent fate for the acyl
phosphate intermediates produced by these enzymes. If the reactions catalyzed by these enzymes follow the same pathway, then the degradation of carboxyphosphate into carbon dioxide and $P_i$ would be paralleled by breakdown of an acyl phosphate into an acylium ion (Scheme 1.4). This would result in an intermediate that is unprecedented in enzyme catalyzed reactions.

Scheme 1.4

**Carboxyltransferase.** In the second half of the reaction catalyzed by acetyl-CoA carboxylase the carboxyl group is transferred from carboxybiotin to acetyl-CoA to form malonyl-CoA. This reaction is catalyzed by the carboxyltransferase component of the multienzyme complex. In contrast to the biotin carboxylase subunit, very few mechanistic studies have been performed on carboxyltransferase. Carboxyltransferase is an $\alpha_2\beta_2$ tetramer and a system capable of overexpressing stoichiometric amounts of each
subunit, that does not form inclusion bodies, only recently has been established (Blanchard and Waldrop, 1998). Prior to this it was difficult to obtain sufficient amounts of protein for kinetic and structural studies.

The overexpression system for carboxyltransferase also provides a convenient method for performing site-directed mutagenesis studies. However, it would be advantageous to mutagenesis studies if the three-dimensional structure of carboxyltransferase were known. Knowing the structure of the protein would provide a guide to determine which residues to mutate. Unfortunately, solving the three-dimensional structure of carboxyltransferase has proven difficult. Although crystals of carboxyltransferase have been grown, they diffract to a very low resolution and have not been useful in solving the enzyme structure. One way to overcome this problem is to perform cocrystallization experiments of carboxyltransferase with substrates. The binding of a substrate to the enzyme may promote the growth of a different crystal form that diffracts to a higher resolution. An enzyme structure with substrates bound also would be useful in determining interactions between active site residues and the substrates. However, cocrystallization of biotin with carboxyltransferase is difficult because the dissociation constant of biotin for carboxyltransferase is in the mM range (Blanchard and Waldrop, 1998). Thus, it is difficult to add enough biotin to the crystallization medium to saturate the enzyme. A possible way to overcome the low affinity of biotin for carboxyltransferase is to make a bisubstrate analog.

Recently Amspacher et al. (1999) described the synthesis of a multisubstrate analog inhibitor of the biotin carboxylase component of acetyl-CoA carboxylase. This
inhibitor was formed by coupling phosphonoacetic acid to the 1’-N position of biotin. Like carboxyltransferase, the dissociation constant of biotin for biotin carboxylase also is in the mM range. Through the synthesis of the multisubstrate analog, the affinity of biotin for biotin carboxylase was increased (Blanchard et al., 1999). Not only does this demonstrate how a multisubstrate analog can increase the dissociation constant between a substrate and enzyme, but Amspacher et al. (1999) also described a convenient method for the functionalization of biotin through the 1’-N atom. The synthesis of the multisubstrate analog of biotin carboxylase paves the way for the synthesis of other biotin analogs, including the possibility of a bisubstrate analog of carboxyltransferase.

**Carboxyltransfer reaction mechanism.** Carboxyl transfer from carboxybiotin to acetyl-CoA can occur through two possible pathways. In a stepwise mechanism, carboxyl transfer would occur subsequent to proton abstraction from the methyl group of acetyl-CoA. Alternatively, the reaction could occur through a concerted mechanism in which proton removal is concurrent with carboxyl transfer. Studies to distinguish between these two possibilities have not been performed with carboxyltransferase. Studies carried out on pyruvate carboxylase, however, have determined that carboxyl transfer occurs through a stepwise mechanism for this enzyme (Attwood, 1995). Since pyruvate carboxylase and carboxyltransferase both catalyze very similar reactions this suggests carboxyltransferase also proceeds through a stepwise mechanism.

**Acetyl-CoA Carboxylase as a Target for Chemotherapeutics**

Aside from serving as a model for biotin-dependent reactions, acetyl-CoA carboxylase has also been recognized as a possible target for chemotherapeutic agents.
This is not surprising given the vital role long-chain fatty acids play in the form and function of cellular membranes, and also as an energy storage for higher eukaryotes. This makes acetyl-CoA carboxylase a target for anti-obesity agents, herbicides, and antibiotics.

Bacteria rely on malonyl-CoA production almost exclusively for the synthesis of fatty acids (Heath et al., 2001). Thus, inhibiting the formation of malonyl-CoA would inhibit the production of fatty acids and lead to cell death. The use of acetyl-CoA carboxylase as a target for antibiotics is supported by studies involving temperature-sensitive mutations in the genes encoding biotin carboxylase (Campbell and Cronan, 2001), the biotin carboxyl carrier protein (Li and Cronan, 1992), and one of the genes of carboxyltransferase (Li et al., 1992). In bacteria that contain one of these mutations the synthesis of fatty acids is blocked at nonpermissive temperatures, demonstrating the essential nature of this enzyme complex. Also, since most biotin-dependent enzymes do not recognize free biotin as a substrate, the use of biotin derivatives as therapeutic agents is feasible. There are two classes of antibiotics currently in use that provide precedent for the use of drugs to block fatty acid synthesis. The isoniazid and triclosan antibiotics specifically block the fatty acid synthase enzyme complex in bacteria (Banerjee et al., 1994; Heath et al., 1998). By inference, antibiotics that target acetyl-CoA carboxylase should have similar effects, and they should prove to be useful therapeutic agents.

Recently there has been a great deal of interest in the development of drugs that block fatty acid synthesis in humans. Obesity is a major health problem that affects over 50% of the American population (Must et al., 1999). Medical problems associated with
obesity include type II diabetes, cardiovascular disease, cerebrovascular disease, and increased mortality (Must et al., 1999). The magnitude of obesity and the severity of the complications associated with obesity emphasize the need for new treatments. A recent study found that mice treated with inhibitors of fatty acid synthase resulted in a decreased appetite and a reduced body weight (Loftus et al., 2000). Loftus et al. suggest the decrease in appetite found in these mice may result from an increase in malonyl-CoA concentrations, which is a result of the decrease in fatty acid synthase activity. Malonyl-CoA, produced by acetyl-CoA carboxylase, feeds into the fatty acid synthase complex where it is used for the synthesis of long chain fatty acids. An increase in malonyl-CoA concentrations may act as a signal for appetite suppression (Loftus et al., 2000). This suggests that fatty acid synthesis may represent a link in feeding regulation, as well as obesity. Mice that have been genetically engineered so that they lack one isoform of acetyl-CoA carboxylase (ACC2) have been shown to lose weight despite eating up to 30% more food than wild-type mice (Abu-Elheiga et al., 2001). Since ACC2 is found primarily in heart, muscle, and liver tissues the researchers suggest that the mice oxidize more fatty acids in these organs, signaling the rest of the body to mobilize fat stores. This demonstrates a convincing link between acetyl-CoA carboxylase and obesity, and identifies acetyl-CoA carboxylase as a target for anti-obesity agents.

Acetyl-CoA carboxylase has been the target of herbicides for many years (Gronwald, 1994). Two classes of herbicides currently in use, the graminicides and the aryloxyphenoxypropionates, inhibit acetyl-CoA carboxylase of grasses, but have no effect on the isoform of this enzyme found in broad leaf species. This makes these
herbicides quite useful for the agricultural industry. However, many of the grasses are becoming resistant to these drugs, and there is a need to develop better inhibitors of acetyl-CoA carboxylase for use as herbicides.

**Purpose of Dissertation**

This dissertation describes three separate studies of acetyl-CoA carboxylase. The first study is a direct test of the proposed hypothesis for the deprotonation of biotin by biotin carboxylase. Previous studies on biotin-dependent carboxylases have indicated that biotin is deprotonated in biotin carboxylase through the activity of the active site residues Cys230 and Lys238. To test the proposed mechanism for the enzymatic deprotonation of biotin, site-directed mutagenesis was performed on these residues, and the mutant enzymes were tested by a number of kinetic assays. The second study describes the synthesis and enzymatic characterization of a bisubstrate analog inhibitor of the carboxyltransferase component of acetyl-CoA carboxylase. The final study shows that treatment of preadipocytes with a precursor to the bisubstrate analog of carboxyltransferase can inhibit the activity of acetyl-CoA carboxylase *in vivo*, and thus prevent adipogenesis in these cells.

**References**


CHAPTER 2

DO CYSTEINE 230 AND LYSINE 238 OF BIOTIN CARBOXYLASE PLAY A ROLE IN THE ACTIVATION OF BIOTIN?*

Introduction

Biotin, or vitamin H, serves as a cofactor for a diverse group of enzymes that catalyze carboxylation reactions. These biotin-dependent carboxylases are involved in many vital metabolic pathways such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism. The reactions catalyzed by this family of enzymes follow the same two-step pathway shown below:

(1) \[ \text{Enzyme-biotin} + \text{MgATP} + \text{HCO}_3^- \xrightarrow{\text{Mg}^{2+}} \text{Enzyme-Biotin-CO}_2^- + \text{MgADP} + P_i \]

(2) \[ \text{Enzyme-biotin-CO}_2^- + \text{Acceptor} \xrightarrow{} \text{Acceptor-CO}_2^- + \text{Enzyme-biotin} \]

The first partial reaction involves the carboxylation of biotin at the N1’ position. This is accomplished by the ATP-dependent phosphorylation of bicarbonate, the source of CO₂, forming a reactive carboxyphosphate intermediate. The carboxyl group is then transferred to biotin, which is covalently linked to the enzyme through an amide linkage to the side chain of a specific lysine residue. In the second half of the reaction the carboxylate group is transferred from carboxybiotin to an acceptor molecule. The acceptor will vary depending on the enzyme. Pyruvate carboxylase, for example, utilizes

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pyruvate as an acceptor while acetyl-CoA carboxylase utilizes acetyl-CoA as its substrate.

The first half reaction, which is catalyzed by all biotin-dependent carboxylases, involves the deprotonation of the 1’ nitrogen of biotin. Mechanistic studies utilizing the biotin carboxylase component of *E. coli* acetyl-CoA carboxylase and pyruvate carboxylase have suggested a possible mechanism for the deprotonation of biotin. Tipton and Cleland found a large inverse solvent isotope effect in the reaction catalyzed by biotin carboxylase, which suggests a sulfhydryl group is involved in a proton transfer (Tipton and Cleland, 1988b). Participation of an active site cysteine residue in catalysis by biotin carboxylase was further supported by the observation that N-ethylmaleimide inactivated the enzyme while the substrates protected against the inactivation (Tipton and Cleland, 1988a). Werneburg and Ash (1993) found that o-phthalaldehyde (o-PA) inactivated pyruvate carboxylase by crosslinking cysteine and lysine residues and that substrates protected against inactivation, suggesting that the modified cysteine and lysine residues were in the active site. All this evidence taken together led several groups (Tipton and Cleland, 1988a,b; Werneburg and Ash, 1993; Attwood *et al.*, 1986; Attwood, 1995) to propose an active site lysine residue removes the proton from the thiol group of a nearby cysteine residue. The thiolate anion then abstracts the N1’ proton of biotin while the protonated lysine residue stabilizes the negative charge on the ureido oxygen (Scheme 2.1).
Using the crystal structure of *E. coli* biotin carboxylase (Waldrop *et al*., 1994), the only three-dimensional structure of a biotin-dependent carboxylase, Jitrapakdee *et al.* proposed that the cysteine and lysine residues acting as acid-base catalysts are Cys230 and Lys238 (Jitrapakdee *et al*., 1996). These residues are located in the active-site of biotin carboxylase and the sulfur atom of Cys230 and ε-amino group of Lys238 are sufficiently close (4.2Å) to allow for crosslinking of these two residues by o-PA. Cys230 and Lys238 are also strictly conserved in all biotin-dependent carboxylases, suggesting they play a role in the catalytic mechanism (Kondo *et al*., 1991).

The biotin carboxylase component of *E. coli* acetyl-CoA carboxylase has been used as a model system for mechanistic studies of biotin carboxylation because this enzyme retains activity when isolated from the multienzyme complex. Moreover, biotin
carboxylase is able to utilize free biotin as a substrate thereby simplifying kinetic analysis (Moss and Lane, 1971). A system for site-directed mutagenesis studies of *E. coli* biotin carboxylase has been described recently which allows for overexpression and purification of mutant forms of biotin carboxylase that are free of contaminating wild-type enzyme derived from the chromosomal copy of the gene (Blanchard et al., 1999). This system was used to test directly the hypothesis that Cys230 and Lys238 act as an acid-base pair to remove the N1’ proton from biotin.

**Experimental Procedures**

**Chemicals and Enzymes.** Primers were synthesized by Life Technologies GibcoBRL. Sodium bicarbonate labeled with $[^{14}\text{C}]$ was from Amersham and had a specific activity of 0.1 mCi/mmol. His-binding resin and restriction grade thrombin were from Novagen. Pyruvate kinase was from Boehringer Mannheim. All other reagents were from Sigma or Aldrich.

**Site-Directed Mutagenesis and Enzyme Purification.** Site-directed mutagenesis and purification of wild-type and mutant enzymes were as previously described by Blanchard *et al.* (1999). Cys230 was mutated to alanine, and Lys238 was mutated to glutamine. The lysine to glutamine substitution is more isosteric than an alanine replacement and the amide side chain cannot act as a base. The pairs of internal primers used to make each site-directed mutant were as follows (the bases that were changed are underlined): For C230A, 5’-GCGGAACGTGACGC[CGCT]CCATGCAACGC-3’ and 3’-CGCCTTGCACCTG[GAAGGT]ACGTTGCG-5’ and for K238Q, 5’-GCCGCCACCCAG[AA]GTTGGTCAAGA-3’ and
3’-CGGCCGTGGTCTTCACCAGCTTTCT-5’. The entire gene of each mutant form of biotin carboxylase was sequenced to verify the mutation and confirm there were no other changes in the sequence.

**Enzyme Assays.** The rate of ATP hydrolysis by biotin carboxylase was determined spectrophotometrically by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase, and following the oxidation of NADH at 340 nm (Blanchard *et al*., 1999). When the concentration of ATP was varied above 2 mM (2 mM to 25 mM), the concentration of MgCl₂ was increased to 50 mM, ensuring the formation of MgATP complex. Solvent deuterium isotope effects on ATP hydrolysis by biotin carboxylase were determined in 80% D₂O and a pD of 8.0, where pD’s were calculated according to the equation pD = pH + 0.4 (Glasoe and Long, 1960). When the concentration of bicarbonate was varied, all solutions were degassed in order to lower the endogenous levels of bicarbonate (Blanchard *et al*., 1999).

The rate of ATP synthesis from ADP and carbamoyl phosphate was determined spectrophotometrically with the coupled enzyme system of hexokinase and glucose-6-phosphate dehydrogenase, where the production of NADPH was followed at 340 nm (Blanchard *et al*., 1999). Data were collected using a Uvikon 810 (Kontron Instruments) spectrophotometer interfaced to a PC equipped with a data acquisition program. Reactions were initiated by the addition of enzyme, and were held at 25 °C by a circulating water bath. Kinetic parameters were calculated per active site using a MW of 50,000 Daltons for the biotin carboxylase monomer (biotin carboxylase exists as a homodimer).
To determine if there was a stoichiometric production of ADP and carboxybiotin, the amount of carboxybiotin produced by biotin carboxylase was determined using a $[^{14}C]$ bicarbonate fixation assay and compared to the production of ADP as described by Blanchard et al. (1999).

**Inactivation by NEM.** Enzyme modification by N-ethylmaleimide (NEM) was performed in 10 mM Hepes, 500 mM KCl, pH 7.0. The reaction was initiated by the addition of NEM to a final concentration of 6 mM. Aliquots were removed at regular time intervals and assayed for residual activity. The reaction velocity of each aliquot, for wild-type enzyme, was determined in 100 mM biotin, 3 mM ATP, 15 mM bicarbonate, and 8 mM MgCl$_2$ at pH 8.0 in 100 mM Hepes. When determining reaction velocities for both mutants, the concentrations of ATP and MgCl$_2$ were increased to 20 mM and 50 mM, respectively. The rate constants for inactivation by NEM were determined by least squares analysis of a plot of $\ln(v/\nu_0)$ versus time, where $v$ is the velocity at time $t$, $\nu_0$ is the initial velocity, and the slope of the line is equal to the negative of the rate constant. The pH dependence of the rate of inactivation was determined by incubating biotin carboxylase with 70 µM NEM in a 100 mM solution of the appropriate buffer. The buffers used and their pH ranges were as follows: Hepes, pH 7.0-8.0; TAPS, pH 8.0-9.0; CHES, pH 9.0-9.5.

**Data Analysis.** The $K_m$ and $k_{cat}$ were determined by fitting the velocity versus substrate concentration data to the Michaelis-Menten equation using the non-linear regression program Enzfitter.
The data for the pH dependence on the rate of inactivation were fitted to the log form of equation 2.1, where $y$ represents the rate of inactivation at a particular pH value, $C$ represents the pH-independent value of the rate constant, $K_a$ is an acid dissociation constant, and $H$ is the hydrogen ion concentration.

$$y = \frac{C}{1 + \frac{H}{K_a}} \quad (2.1)$$

Results

Bicarbonate-Dependent ATP Hydrolysis. Biotin carboxylase from *E. coli* catalyzes a slow bicarbonate-dependent ATP hydrolysis reaction in the absence of biotin (Climent and Rubio, 1986) (reaction 3):

$$\text{(3) } \text{MgATP} + \text{H}_2\text{O} \xrightarrow{\text{HCO}_3^-} \text{MgADP} + \text{P}_i$$

Current evidence suggests this reaction proceeds through the formation of carboxyphosphosphate, which rapidly decomposes in the absence of biotin (Ogita and Knowles, 1988). The Michaelis constants for ATP and bicarbonate and the $k_{cat}$ of this partial reaction were determined for the wild-type enzyme and both mutants of biotin carboxylase (Table 2.1). The $k_{cat}$ for the C230A and K238Q mutants were equivalent to the $k_{cat}$ for wild-type biotin carboxylase. In contrast, a 50-fold increase in the Michaelis constant for ATP was observed for both mutant enzymes.

The Michaelis constant for bicarbonate was determined at fixed, non-saturating levels of ATP and biotin.$^1$ Therefore, the $K_m$ values for bicarbonate reported here are apparent $K_m$ values. The apparent $K_m$ values for bicarbonate with either the wild-type
**Table 2.1** Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m^{\text{ATP}}$ (mM)</th>
<th>$K_m^{\text{HCO}_3}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.073 ± 0.001$^\dagger$</td>
<td>0.081 ± 0.003$^\dagger$</td>
<td>0.37 ± 0.04$^\dagger$</td>
</tr>
<tr>
<td>C230A</td>
<td>0.102 ± 0.001</td>
<td>4.23 ± 0.13</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>K238Q</td>
<td>0.117 ± 0.002</td>
<td>6.87 ± 0.27</td>
<td>*</td>
</tr>
</tbody>
</table>

*Refer to text for results. The $k_{\text{cat}}$ and $K_m$ values for ATP were determined by varying ATP at a constant saturating level of bicarbonate. The $K_m$ value for bicarbonate for the C230A mutant was determined by varying bicarbonate at a constant nonsaturating level of ATP (20 mM) and biotin (75 mM). The standard errors on $k_{\text{cat}}$ and $K_m$ were determined from the non-linear regression analysis. $^\dagger$These values were taken from Blanchard et al., 1999.
biotin carboxylase or the C230A mutant were similar. However, for K238Q there was no change in the reaction rate upon increasing concentrations of bicarbonate (0.5 mM to 7.1 mM). This may be due to a decrease in the Michaelis constant for bicarbonate to a value lower than the concentration of bicarbonate that remained in the degassed buffer. Thus, the endogenous level of bicarbonate (0.5 mM at pH 8.0; Asada, 1982) was enough to saturate the enzyme.

**Biotin-Dependent ATP hydrolysis.** The rate of ATP hydrolysis by wild-type biotin carboxylase is significantly increased in the presence of biotin. The initial velocity of ATP hydrolysis as a function of increasing amounts of biotin for wild-type biotin carboxylase and the two mutants is shown in Figure 2.1. The $k_{cat}$ of the biotin-dependent ATPase for the wild-type enzyme was $73.8 \pm 3.8$ min$^{-1}$, which is 1000-fold faster than the $k_{cat}$ of the rate of ATP hydrolysis in the absence of biotin (Table 2.2). The $k_{cat}$ for the C230A mutant was $30.8 \pm 1.6$ min$^{-1}$, slightly less than 50% the velocity for the wild-type enzyme. The Michaelis constant for biotin also was relatively unaffected by the C230A mutation. Wild-type biotin carboxylase and the C230A mutant had a $K_m$ for biotin of $122 \pm 17$ mM and $167 \pm 18$ mM, respectively. In contrast to wild-type biotin carboxylase and the C230A mutant, the K238Q mutation had a drastic effect on the biotin-dependent ATP hydrolysis reaction. As can be seen in Figure 2.1, increasing concentrations of biotin had very little effect on the rate of ATP hydrolysis for the K238Q mutant. The reaction rate was equivalent to the $k_{cat}$ for the bicarbonate-dependent ATPase reaction (Table 2.1).
Figure 2.1 Effect of biotin on the ATP hydrolysis reaction for wild-type biotin carboxylase (WT) and both mutants of biotin carboxylase, C230A and K238Q. Initial velocities were measured at a saturating level of ATP and bicarbonate with increasing levels of biotin. The initial velocities represent the velocity divided by the concentration of active sites. The points are the experimental velocities and the lines for the wild-type and C230A mutant are derived from the best fit of the data to the Michaelis-Menten equation.


**Table 2.2** Kinetic Parameters for the Biotin-Dependent ATPase Reaction

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$73.8 \pm 3.8^\dagger$</td>
<td>$122.4 \pm 16.9^\dagger$</td>
</tr>
<tr>
<td>C230A</td>
<td>$30.8 \pm 1.6$</td>
<td>$167.5 \pm 17.9$</td>
</tr>
<tr>
<td>K238Q</td>
<td>$0.117 \pm 0.002$</td>
<td>*</td>
</tr>
</tbody>
</table>

*Refer to text for results. The $k_{cat}$ and $K_m$ values for biotin were determined by varying biotin at a constant saturating level of bicarbonate and ATP. The standard errors on $k_{cat}$ and $K_m$ were determined from the non-linear regression analysis. $^\dagger$These values were taken from Blanchard *et al.*, 1999.
It should be noted that a previous study of the K238Q mutant enzyme found the maximal velocity was decreased only 4-fold while the $K_m$ for biotin was equivalent to that observed for the wild-type enzyme (Kazuta et al., 1998). A possible explanation for the difference between the findings of Kazuta et al. and the results reported here is that Kazuta et al. did not demonstrate a method for separating the mutant enzyme from the wild-type enzyme derived from the chromosomal copy of the bacterial genome. Thus, the maximal velocity and Michaelis constant for biotin that were reported for the K238Q mutant were probably the result of contaminating wild-type biotin carboxylase.

The assay for the biotin-dependent hydrolysis of ATP measured the rate of production of ADP using the coupling enzymes pyruvate kinase and lactate dehydrogenase. The C230A and K238Q mutations clearly did not affect the ability of biotin carboxylase to hydrolyze ATP. This method, however, did not measure the ability of biotin carboxylase to transfer the carboxyl group from carboxyphosphate to biotin. An assay using radiolabeled bicarbonate was used to measure the amount of carboxybiotin produced by wild-type biotin carboxylase and the two mutant enzymes. The amount of ADP was also measured to determine if there was a stoichiometric formation of ADP and carboxybiotin. The ratio of the micromoles of carboxybiotin formed to the micromoles of ADP formed for wild-type, C230A, and K238Q was 1.00, 1.07, and zero, respectively. The wild-type enzyme and the C230A mutant both demonstrated a 1:1 stoichiometry for the production of ADP and carboxybiotin. Thus, the C230A mutation had no effect on the ability of biotin carboxylase to produce carboxybiotin. Although the K238Q mutant was able to hydrolyze ATP, it did not produce a measurable quantity of carboxybiotin.
This suggested the production of carboxybiotin had been uncoupled from ATP hydrolysis by this mutation. Alternatively, biotin may not have been able to bind to the K238Q mutant enzyme. To distinguish between these two possibilities another model reaction catalyzed by biotin carboxylase was examined.

**ATP Synthesis Reaction.** Biotin carboxylase from *E. coli* will catalyze an ATP synthesis reaction (reaction 4):

\[
\text{(4) MgADP + Carbamoyl-P} \rightleftharpoons \text{MgATP + CO}_2 + \text{NH}_3
\]

In this reaction, a phosphate group is transferred from carbamoyl phosphate to ADP forming ATP and carbamate, which rapidly decomposes into carbon dioxide and ammonia. This reaction is believed to represent the reverse reaction, with carbamoyl phosphate acting as a substrate analog of the putative carboxyphosphate intermediate (Polakis *et al.*, 1974). The rate of this reaction is increased in the presence of biotin, which does not participate in the chemistry of the reaction but is thought to activate the enzyme via a conformational change (Polakis *et al.*, 1974). Thus, the degree of activation of the reaction by biotin can be used to assess whether biotin binds to the K238Q mutant enzyme. First, however, the effect of the two mutations on the Michaelis constants for carbamoyl phosphate and ADP and the $k_{\text{cat}}$ of the reaction in the absence of biotin were determined (Table 2.3). The mutations did not have a significant effect on either the $k_{\text{cat}}$ of the reaction or the binding of carbamoyl phosphate. In addition, only a moderate effect on the $K_m$ of ADP was observed. It is important to note that biotin carboxylase will catalyze the ATP synthesis reaction in the absence of biotin. Though it has been
**Table 2.3** Kinetic Parameters for the ATP Synthesis Reaction

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (ADP) (mM)</th>
<th>$K_m$ (CbmP) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>0.31 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>4.84 ± 0.24</td>
</tr>
<tr>
<td><strong>C230A</strong></td>
<td>0.42 ± 0.01</td>
<td>0.62 ± 0.05</td>
<td>7.94 ± 0.31</td>
</tr>
<tr>
<td><strong>K238Q</strong></td>
<td>0.43 ± 0.01</td>
<td>1.2 ± 0.1</td>
<td>3.13 ± 0.27</td>
</tr>
</tbody>
</table>

The kinetic parameters were determined by varying carbamoyl phosphate (CbmP) at a constant saturating level of ADP or varying ADP at a constant saturating level of CbmP. The standard errors on $k_{cat}$ and $K_m$ were determined from the non-linear regression analysis.
previously reported that biotin is absolutely required for ATP synthesis activity (Polakis et al., 1974), our results have shown this is not correct. Biotin simply stimulates the ATP synthesis activity.

To evaluate the level of stimulation of this reaction by biotin for wild-type biotin carboxylase and the two mutants, initial velocities were measured at a saturating concentration of ADP and carbamoyl phosphate, both in the absence of biotin and in the presence of 64 mM biotin. The degree of stimulation by biotin in the C230A mutant was similar to that observed in the wild-type enzyme, a 22.8 ± 0.9-fold and 19.8 ± 2.0-fold increase, respectively. This is consistent with the fact that the C230A mutation did not significantly affect the Michaelis constant for biotin. The rate of ATP synthesis catalyzed by K238Q was stimulated 1.50 ± 0.03-fold. Although this degree of stimulation by biotin was small compared to wild-type, it suggested biotin was able to bind to the K238Q mutant enzyme.

**Solvent Deuterium Isotope Effects.** While the site-directed mutagenesis studies were a direct test of whether Cys230 and Lys238 were involved in removing the proton from the 1’ nitrogen of biotin, it was of interest to see what effect these two mutations had on the solvent isotope effect and chemical inactivation by N-ethylmaleimide observed in the wild-type enzyme. The inverse solvent isotope effect and chemical inactivation by N-ethylmaleimide for wild-type biotin carboxylase were two of the principal pieces of evidence suggesting a cysteine was involved in the catalytic mechanism (Tipton and Cleland, 1988a,b).
Biotin carboxylase from *E. coli* exhibits an inverse solvent isotope effect on both the $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ parameters (Tipton and Cleland, 1988b). Since thiol groups have inverse fractionation factors, Tipton and Cleland proposed a catalytic mechanism involving a cysteine residue undergoing proton exchange (Tipton and Cleland, 1988b). We have confirmed there is an inverse kinetic solvent isotope effect on the $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ parameters of the biotin-dependent ATPase reaction for the wild-type enzyme (Table 2.4), and we have extended the analysis by measuring the solvent isotope effect on the bicarbonate-dependent ATPase reaction. If the origin of the inverse solvent isotope effect was due solely to a cysteine residue removing the proton from the 1’ nitrogen of biotin, then an inverse solvent isotope effect would not be observed in the bicarbonate-dependent ATPase reaction because biotin is not involved in this reaction. However, as shown in Table 2.4, an inverse solvent isotope effect on both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ was observed for the bicarbonate-dependent ATPase reaction for wild-type biotin carboxylase. Thus, the solvent isotope effect remained inverse in a reaction that did not involve biotin. Since the bicarbonate-dependent ATPase reaction is the first part of the entire reaction involving biotin, the data suggest the source of the inverse solvent isotope effect in the biotin-dependent ATPase reaction may solely or in part be due to the inverse kinetic solvent isotope effect of the bicarbonate-dependent ATPase reaction. Thus, an inverse kinetic solvent isotope effect should not be used to infer a role for cysteine in the deprotonation of biotin.
Table 2.4 Solvent Deuterium Isotope Effects

<table>
<thead>
<tr>
<th></th>
<th>Biotin dependent ATPase</th>
<th>HCO₃⁻ dependent ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D(kₐₑₜ)</td>
<td>D'(kₐₑₜ/Kₘ)</td>
</tr>
<tr>
<td>WT</td>
<td>.63 ± .08</td>
<td>.45 ± .10</td>
</tr>
<tr>
<td>C230A</td>
<td>1.07 ± .12</td>
<td>1.15 ± .40</td>
</tr>
<tr>
<td>K238Q</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Solvent isotope effects were determined in 80% D₂O. For the biotin-dependent ATPase reaction, biotin was varied for the wild-type enzyme at saturating levels of ATP (3 mM) and bicarbonate (15mM), while for the C230A mutant ATP was held constant at 20 mM and bicarbonate was saturating at 15 mM. For the bicarbonate-dependent ATPase reaction, ATP was varied at a saturating concentration of bicarbonate (15 mM). The error was calculated by standard propagation of the errors from the kₐₑₜ and kₐₑₜ/Kₘ.

*The concentration of biotin did not have an affect on the rate of ATP hydrolysis by the K238Q mutant.
Since studies of enzyme catalyzed reactions performed in D₂O result in protic exchanges at many sites on both the enzyme and substrate, solvent isotope effects are often difficult to interpret. Thus, the cause of the inverse solvent isotope effect observed for biotin carboxylase is not readily apparent. Proton exchange involving a thiol group is not the only explanation for inverse solvent isotope effects. For some enzymes, including thermolysin (Stein, 1988) and carbonic anhydrases (Kassebaum and Silverman, 1989), inverse solvent isotope effects have been attributed to metal ions. In addition, it has been suggested low-barrier hydrogen bonds might also result in isotope effects of less than one (Cleland, 1992). At this time it is difficult to suggest a cause for the solvent isotope effects observed in biotin carboxylase, but it is unlikely they arise from a proton exchange involving the 1’ nitrogen of biotin.

Table 2.4 also shows the kinetic solvent isotope effects for both mutants of biotin carboxylase. In the biotin-dependent ATPase reaction the C230A mutant had, within error, a kinetic solvent isotope effect of unity on both kinetic parameters. This change in solvent isotope effect brought about by the C230A mutation suggests that Cys230 may be responsible for the inverse kinetic solvent isotope effect. However, an alternative interpretation consistent with all of the data is the mutation caused a change in the rate-determining step of the reaction, so that the isotope sensitive step is no longer rate-limiting. For the bicarbonate-dependent ATPase reaction, neither mutation had an effect on the kinetic solvent isotope effect on the $k_{\text{cat}}/K_m$ parameter. In contrast, the kinetic solvent isotope effect on the $k_{\text{cat}}$ for the bicarbonate-dependent ATPase reaction was normal for both C230A and K238Q. A change in the isotope effect on the $k_{\text{cat}}$ but not on
the $k_{\text{cat}}/K_m$ suggests both mutations have resulted in a change in the ratio to catalysis. This means that the rate of a non-isotope sensitive step that occurs after the first irreversible step (e.g. product release) has become rate-limiting.

**Inactivation By NEM.** N-Ethylmaleimide (NEM) is often used to inactivate enzymes presumably by reacting with the thiol group of cysteine residues. Tipton and Cleland found that *E. coli* biotin carboxylase is inactivated by NEM, and the inactivation is blocked by the presence of all three substrates (Tipton and Cleland, 1988a). The inactivation of biotin carboxylase by NEM was one of the principal pieces of evidence implicating a cysteine in the active site of biotin carboxylase. The crystal structure of biotin carboxylase revealed Cys230 as the only cysteine in the active-site of the enzyme (Waldrop *et al.*, 1994), which suggested this residue as the site of NEM modification. This hypothesis was tested by determining the rate of inactivation by NEM for both the C230A and K238Q mutants. If NEM is reacting with Cys230, then the C230A mutant should show little to no inactivation, whereas the K238Q mutant should be inactivated by NEM. The rate of inactivation by NEM of wild-type biotin carboxylase and the two mutants, C230A and K238Q, is shown in Figure 2.2. The C230A mutant was inactivated by NEM at a rate comparable to that of the wild-type enzyme, 0.045 ± 0.002 min$^{-1}$ and 0.060 ± 0.004 min$^{-1}$, respectively. Surprisingly, the K238Q mutant was not rapidly inactivated by NEM. There was a slow loss of activity observed for the K238Q mutant, which could be attributed to the precipitation of the enzyme that occurred in the presence of NEM and not by active-site modification. These results suggest that NEM may be reacting with Lys238 and not Cys230. Even though NEM reacts preferentially with
Figure 2.2 Inactivation by N-ethylmaleimide (NEM) for wild-type biotin carboxylase (WT) and both mutants of biotin carboxylase, C230A and K238Q. Each enzyme was incubated with 6 mM NEM at pH 7.0. Aliquots were removed at regular time intervals and assayed for remaining activity. The residual activity is expressed as the percent activity compared to the activity at time zero.
cysteine residues, it has been shown to react with lysine as well (Brewer and Riehm, 1967). Moreover, modification of Lys238 by NEM and not Cys230 is consistent with the observation that the K238Q mutation results in a dramatic decrease in enzyme activity, whereas mutation of Cys230 affects biotin carboxylase activity only slightly.

The modification of Lys238 by NEM provides a means to measure the pK of Lys238 by determining the pH dependence of the rate of inactivation. The pH dependence of the rate of inactivation of biotin carboxylase is shown in Figure 2.3. It was not possible to measure the rate of inactivation higher than pH 9.5 because of the loss of enzyme activity. Because a complete pH profile could not be obtained, the pK value can only be estimated to be 9.4 or higher. This high pK value suggests Lys238 is protonated at physiological pH, and carries a positive charge at the onset of the enzymatic reaction.

**Discussion**

The objective of this study is to determine whether Cys230 and Lys238 act in concert to remove the proton from the 1’ nitrogen of biotin. The results of the bicarbonate-dependent ATPase assay are consistent with the proposed roles of Cys230 and Lys238 in the catalytic mechanism of biotin carboxylase. The fact that neither mutation affected the $k_{cat}$ of the bicarbonate-dependent ATP hydrolysis assay suggests that neither Cys230 nor Lys238 is essential for formation of the carboxyphosphate intermediate. Biotin does not participate in the bicarbonate-dependent ATP reaction. Therefore, residues that are supposed to interact with biotin would not be essential to the
Figure 2.3 Inactivation of wild-type biotin carboxylase by N-ethylmaleimide (NEM) as a function of pH. The rate of inactivation was determined by incubating biotin carboxylase with 70 µM NEM at increasing pH values. The data points represent the experimentally determined inactivation rates and the line is the best fit of the data to the log form of equation 2.1.
bicarbonate-dependent ATPase activity. However, the increase in $K_m$ values for ATP for both C230A and K238Q compared to wild-type suggest these residues are involved in binding ATP, either directly or indirectly. In fact, Artymiuk et al. postulated that Lys238 interacted with ATP based on a comparison of the three-dimensional structures of biotin carboxylase and D-Ala:D-Ala ligase (Artymiuk et al., 1996). Biotin carboxylase and D-Ala:D-Ala ligase were found to have very similar three-dimensional structures despite having very little sequence homology. The common feature between biotin carboxylase and D-Ala:D-Ala ligase is they catalyze an ATP-dependent ligation of a carboxyl group to a nitrogen atom via an acyl phosphate intermediate. The crystal structure of D-Ala:D-Ala ligase contained a substrate analog bound at the active site that allowed the interactions between the analog and protein to be determined. The D-Ala:D-Ala ligase structure suggested the side chain amine of the residue equivalent to Lys238 in D-Ala:D-Ala ligase, Lys215, was bound to the $\gamma$-phosphate of ATP (Fan et al., 1994). This is consistent with affinity labeling of biotin carboxylase with adenosine diphosphopyridoxal, which also suggested Lys238 interacts with the $\gamma$-phosphate of ATP (Kazuta et al., 1998).

While the results of the bicarbonate-dependent ATPase assay are consistent with the proposed roles of Cys230 and Lys238, the results of the biotin-dependent ATPase assay argue strongly against Cys230 and Lys238 acting in concert to remove the proton from the 1’ nitrogen of biotin. The data from the biotin-dependent ATPase assay suggest that Cys230 is not an essential residue in the catalytic mechanism of biotin carboxylase because the $k_{cat}$ of this reaction is not drastically affected by the C230A mutation. Also,
the lack of a significant change in the $K_m$ for biotin for the C230A mutant suggests Cys230 does not have any interaction with biotin. Most importantly, if Cys230 acted as a catalytic base to remove the 1’ nitrogen of biotin to allow for carboxylation, then very little carboxybiotin should be formed compared to ATP hydrolyzed. A 1:1 ratio for the production of ADP and carboxybiotin clearly showed that the C230A mutation does not diminish the ability of biotin carboxylase to carboxylate biotin. Thus, it is very unlikely Cys230 acts as a catalytic base to remove the proton from the 1’ nitrogen of biotin.

In contrast to the C230A mutation, the K238Q mutation had a significant effect on the ability of biotin carboxylase to produce carboxybiotin. While the K238Q mutant retained the ability to hydrolyze ATP in the presence of bicarbonate at a rate equivalent to wild-type (Table 2.1), there was no increase in the rate of ATP hydrolysis in the presence of biotin, as well as no detectable formation of carboxybiotin. Stimulation of the ATP synthesis activity by biotin suggested that biotin could bind to the K238Q mutant. However, the small degree of activation of the ATP synthesis reaction in the K238Q mutant may indicate the affinity of biotin for biotin carboxylase has been significantly decreased by the K238Q mutation. All of these results strongly suggest that Lys238 plays a critical role in carboxyl transfer from carboxyphosphate to biotin. This leads to the question of whether Lys238 might directly abstract the N1’ proton of biotin. But the pH dependence of the inactivation by NEM suggests this residue has a pK of 9.4 or greater, which makes it an unlikely candidate for a catalytic base. Consequently, all of the data taken as a whole indicate that Cys230 and Lys238 do not act as a pair of acid-base catalysts to remove the proton from the 1’ nitrogen of biotin.
Although Lys238 may not be a catalytic base, the data show that it does play an important role in the transfer of the carboxyl group from carboxyphosphate to biotin. Any postulated role for Lys238 in the catalytic mechanism must be consistent with the following observations: (1) Lys238 interacts with the γ-phosphate of ATP, (2) Lys238 has an interaction with biotin, and (3) Lys238 carries a positive charge. A possible role for Lys238 in the catalytic mechanism of biotin carboxylase stems from the work of Shi and Walsh (Shi and Walsh, 1995). They mutated the lysine residue equivalent to Lys238 in D-Ala:D-Ala ligase, Lys215, and concluded that the protonated ε-amino group interacted with the γ-phosphate of ATP and helped to orient the γ-phosphate for transfer to d-alanine. In an analogous manner for biotin carboxylase, Lys238 interacts with the γ-phosphate of ATP and in turn the phosphate group of carboxyphosphate, either through an electrostatic interaction or via hydrogen-bonding, and helps to orient the carboxyphosphate intermediate for carboxyl transfer to biotin. Concurrently, the positively charged lysine residue could interact with the carbonyl oxygen of biotin, again either through a hydrogen bond or an electrostatic interaction. This would promote the tautomerization of the biotin molecule to the imidate form, which is more nucleophilic than the ureido form (Hegarty et al., 1969).

Finally, since neither Cys230 nor Lys238 act as a catalytic base to remove the proton from the 1’ nitrogen of biotin, the question remains as to how the proton from the 1’ nitrogen of biotin is removed? Perhaps an active site amino acid is not involved at all. An alternative hypothesis begins with the observation that transfer of the carboxyl group from carboxyphosphate to biotin requires one of the phosphate oxygens of
carboxyphosphate to be protonated. Thus, one of the phosphate oxygens of carboxyphosphate may act as a catalytic base to abstract the proton from the 1’ nitrogen of biotin. This is a very economical mechanism and similar substrate-assisted catalytic mechanisms have been proposed for aspartate transcarbamylase (Gouaux et al., 1987) and the GTPase activity of the protein ras (Schweins et al., 1995).

Endnotes

1 Biotin was added to increase the initial velocity, which allowed less enzyme to be added to the assay mixture. It was necessary to add a small volume of enzyme to minimize the endogenous level of bicarbonate. The endogenous level of bicarbonate at pH 8.0 is 0.5 mM (Asada, 1982), which is close to the Michaelis constant for bicarbonate in biotin carboxylase. This makes it difficult to vary the level of bicarbonate. The endogenous level of bicarbonate could be removed from all the components of the assay except the enzyme. Hence, it was necessary to minimize the amount of enzyme added.

2 This conclusion is valid if the reaction of bicarbonate and ATP is rate-limiting for the overall catalysis. To the extent that the isotope effects for the bicarbonate-dependent ATPase reaction and the overall reaction with biotin were very similar, the simplest explanation is that the reaction of bicarbonate and ATP to form carboxyphosphate is rate-limiting for the overall catalysis. If this were not the case then one would have to postulate a more complicated mechanism involving two different proton transfers that both happen to give the same isotope effect.

3 The ratio to catalysis is the sum of the ratios of the rate constant for the isotope-sensitive step to the net rate constants for each of the other forward steps (Northrop, 1982; Cook and Cleland, 1981).

References


CHAPTER 3

A BISUBSTRATE ANALOG INHIBITOR OF THE CARBOXYLTRANSFERASE COMPONENT OF ACETYL-CoA CARBOXYLASE*

Introduction

The biosynthesis of fatty acids is an essential metabolic process for all animals, plants, and bacteria. The generation of fatty acids is necessary for lipid membrane biogenesis, and in higher eukaryotes also for energy storage. Given the vital role fatty acids play in the structural integrity of the cell and in metabolism, it is not surprising the enzymes responsible for their synthesis are targets for anti-obesity agents, herbicides, and antibiotics. For example, proteins of the bacterial fatty acid synthase complex are the targets for several antibiotics currently in use, including isoniazid (Banerjee et al., 1994) and triclosan (Heath et al., 1998), while the human enzyme complex is a target for anti-obesity agents (Loftus et al., 2000). Acetyl-CoA carboxylase (ACC), which catalyzes the first committed step in the synthesis of fatty acids, has been the target of herbicides for a number of years (Gronwald, 1994), and there is a renewed interest in bacterial ACC as a target for antibiotics (Campbell and Cronan, 2001; Heath et al., 2001). ACC has also been identified as a target for anti-obesity drugs, in fact mice lacking the gene coding for the mitochondrial isoform of the enzyme have been shown to lose weight despite eating more food (Abu-Elheiga et al., 2001). In this report we describe the synthesis of a

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bisubstrate analog inhibitor of ACC, which will serve as a tool for mechanistic and structural studies of this enzyme.

The reaction catalyzed by ACC utilizes biotin as a cofactor and proceeds through the following two-step mechanism:

\[
\begin{align*}
(1) \quad \text{Enzyme-biotin} + \text{HCO}_3^- + \text{MgATP} & \rightleftharpoons \text{Enzyme-biotin-CO}_2^- + \text{ADP} + \text{P}_i \\
(2) \quad \text{Enzyme-biotin-CO}_2^- + \text{Acetyl-CoA} & \rightleftharpoons \text{Malonyl-CoA} + \text{Enzyme-biotin}
\end{align*}
\]

The first-half reaction is carried out by the biotin carboxylase component of ACC and involves the ATP-dependent carboxylation of biotin with bicarbonate serving as the source of CO\(_2\). The carboxyl group is transferred from the 1'-N of biotin to acetyl-CoA to form malonyl-CoA in the second half reaction, which is catalyzed by carboxyltransferase. For both half-reactions, biotin remains covalently linked to the enzyme through an amide bond to a specific lysine residue of the biotin carboxyl carrier protein, and is designated as enzyme-biotin in the reaction scheme. In *Escherichia coli*, biotin carboxylase, carboxyltransferase, and the biotin carboxyl carrier protein are all separate proteins, while mammalian ACC contains all three components within a single polypeptide (Tanabe et al., 1975).

The *E. coli* form of the enzyme has served as a model system for mechanistic studies of ACC because both biotin carboxylase and carboxyltransferase retain activity when isolated. Moreover, biotin carboxylase and carboxyltransferase will recognize free biotin as a substrate, eliminating the need for the biotin carboxyl carrier protein during
kinetic studies (Moss and Lane, 1971). In recent years most mechanistic studies of ACC have focused on the biotin carboxylase component because the gene for the enzyme has been cloned and overexpressed (Li and Cronan, 1992a; Blanchard et al., 1999). Most importantly, the three dimensional structure of biotin carboxylase has been determined by x-ray crystallography (Waldrop et al., 1994; Thoden et al., 2000). In contrast, very little work has focused on the carboxyltransferase component. Although the genes (carboxyltransferase is an $\alpha_2\beta_2$ tetramer) for carboxyltransferase have been cloned (Li and Cronan, 1992b), a system for the overexpression of the genes has been developed only recently (Blanchard and Waldrop, 1998). The overexpression system now provides an ample supply of protein for structure/function studies of carboxyltransferase.

Cocrystallization experiments of carboxyltransferase with substrates would greatly aid structure-based drug design efforts. However, the dissociation constant of biotin for carboxyltransferase is in the mM range. Thus, it is difficult to add enough biotin to the crystallization medium to saturate the enzyme. A possible way to overcome the low affinity of biotin for carboxyltransferase is to make a bisubstrate analog.

Steady-state kinetic studies of recombinant carboxyltransferase showed a sequential mechanism where both substrates must bind to the enzyme before catalysis can occur (Blanchard and Waldrop, 1998). The initial velocity patterns were consistent with an equilibrium-ordered kinetic mechanism, with malonyl-CoA binding before biotin (Blanchard and Waldrop, 1998). Based on these initial kinetic studies, we postulated that a bisubstrate analog could act as an inhibitor of carboxyltransferase. Bisubstrate analogs have been shown to be inhibitors of enzyme-catalyzed reactions, with the classic
examples being N-phosphonacetyl-L-aspartate (PALA), an inhibitor of aspartate transcarbamylase (Collins and Stark, 1971), and Ap5A, an inhibitor of adenylate kinase (Bone et al., 1986). Since these two inhibitors have proven invaluable in mechanistic and structural studies of the enzymes they inhibit, a bisubstrate analog inhibitor of carboxyltransferase should prove similarly useful. The structure of a bisubstrate analog (1) of carboxyltransferase is shown in Figure 3.1 along with the substrates malonyl-CoA and biotin. The synthesis (Figure 3.2) and inhibitory properties of 1 on the carboxyltransferase component of E. coli ACC are described here.

Experimental Procedures

Materials. His-binding resin was from Novagen. HPLC was performed using a Waters HPLC system equipped with a Waters 996 photodiode array detector. Preparative HPLC used a Waters AP-1 glass column (1 cm x 20 cm) packed with Bondapak C-18, 15-20 µm, purchased from Waters. All NMR spectra were recorded on Bruker (Billerica, MA) spectrometers; 400 MHz for 1H and 31P NMR spectra, and 500 MHz for 13C NMR spectra. All other reagents were from Sigma or Aldrich.

Synthesis of Bisubstrate Analog. Synthesis of compound 2 was as previously described (Amspacher et al., 1999). Compound 1 was synthesized by reacting 2 with coenzyme A. Coenzyme A (1 equivalent) was dissolved in a solution of 1 M triethylammonium bicarbonate buffer (50 ml/mmol, pH 8.5) then added to a solution of 2 (5 equivalents), which was dissolved in MeOH (20 ml/mmol) that was freshly degassed by bubbling with N2. The mixture was stirred under N2 and allowed to react for 12 hours. The reaction mixture was concentrated in vacuo to remove MeOH. The remaining
Figure 3.1 Structures of malonyl-CoA, biotin, and the bisubstrate analog inhibitor 1.
Figure 3.2 Schematic showing the alkylation reaction between Coenzyme A and the chloroacylated biotin analog 2, forming the bisubstrate analog inhibitor 1. See Materials and Methods for details.
aqueous solution was diluted with H₂O to ~15 ml and was extracted with ethyl acetate (2 x 15 ml) to remove unreacted 2. The remaining solution was lyophilized, and then purified by HPLC. A mobile phase of 10 mM KH₂PO₄, pH 6.7 (A) and MeOH (B) was used. The flow rate was 2 ml/min and the gradient was as follows: 100% A at zero time, followed by a linear gradient to 30% B over 60 min, hold at 30% B for 60 min followed by a linear gradient to 100% B over the next 20 min. In this gradient the retention time for 1 was generally 125 to 130 min. Compound 1 was lyophilized to dryness and quantified by UV absorption at 259.5 nm on the basis of the extinction coefficient (16,800 M⁻¹cm⁻¹) for Coenzyme A. ¹H NMR (D₂O) δ 8.57, (s, 1H), 8.27, (s, 1H), 7.44, (s, 5H), 6.18, (d, J = 7.1 Hz, 1H), 5.19, (m, 2H), 4.58, (s, 1H), 4.24, (s, 2H), 4.04, (m, 1H), 3.99, (s, 2H), 3.86, (s, 2H), 3.83, (s, 1H), 3.80, (m, 1H), 3.56, (m, 1H), 3.48, (m, 2H), 3.39, (m, 2H), 3.30, (m, 1H), 3.09 – 2.96, (m, 2H), 2.75, (t, J = 6.5 Hz, 2H), 2.47, (m, 4H), 1.94, (s, 1H), 1.68 – 1.20, (m, 6H), 0.90, (s, 3H), 0.75, (s, 3H); ¹³C NMR (D₂O) δ 176.97, 175.31, 174.45, 172.10, 157.76, 156.20, 153.38, 149.87, 140.37, 136.25, 129.36, 128.98, 119.22, 87.14, 84.31, 74.79, 74.39, 72.43, 67.41, 66.18, 62.57, 60.96, 58.48, 55.86, 55.19, 52.72, 39.21, 38.85, 37.75, 35.99, 34.98, 34.27, 31.66, 28.15, 27.78, 24.54, 21.47, 18.69, 8.84; ³¹P NMR (D₂O) δ 3.04, -11.84, -12.26. Matrix assisted laser desorption ionization MS calculated for [C₄₀H₅₈N₉O₂₀P₃S₂ + K⁺] 1180.08, found 1180.03; also found 1218.85, 1256.92, and 1294.92, which correspond to adducts containing 2, 3, and 4 potassium ions, respectively (See appendix A).

**Enzyme Assays.** Carboxyltransferase was purified from a strain of *E. coli*, which overexpresses the genes coding for the α and β subunits of the enzyme (Blanchard and
Waldrop, 1998). However, there was a slight modification to the previously published procedure. In this study bacteria were grown to saturation at 37 °C in 1 L of LB medium in 2 L flasks, and then induced with 1 g of lactose and incubated without shaking at 25 °C for 24 h. The concentration of carboxyltransferase was determined by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Carboxyltransferase was assayed in the reverse direction, in which the production of acetyl-CoA was detected by using the coupling enzymes malate dehydrogenase and citrate synthase, and the reduction of NAD$^+$ was measured spectrophotometrically (Blanchard and Waldrop, 1998; Guchhait et al., 1975). The bisubstrate analog did not inhibit the activity of either coupling enzyme at 0.6 mM, which is three times higher than the highest concentration used during the inhibition studies. This was determined by measuring the reduction of NAD$^+$ upon addition of a subsaturating concentration of acetyl-CoA (in place of carboxyltransferase) to a reaction mixture, both with and without inhibitor present. There was no decrease in activity observed in the presence of 0.6 mM 1, indicating that neither coupling enzyme was inhibited.

**Data Analysis.** Data for competitive and noncompetitive inhibition were fitted to Eqs. [3.1] and [3.2], respectively, using the programs of Cleland (Cleland, 1979). In Eqs. [3.1] and [3.2], $v$ is the initial velocity, $V_m$ is the maximal velocity, $A$ is the substrate concentration, $I$ is the concentration of inhibitor, $K_m$ is the Michaelis constant, $K_{is}$ is the slope inhibition constant, and $K_{ii}$ is the intercept inhibition constant.

\[
v = V_m \cdot \frac{A}{K_m (1 + I / K_{is})} + A \]  
\[ v = V_m \cdot \frac{A}{K_m (1 + I / K_{is}) + A (1 + I / K_{ii})} \]  

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Results and Discussion

The bisubstrate analog (1) was synthesized by covalently linking Coenzyme A to the 1’-N position of biotin via an acyl bridge (Figure 3.2). The synthesis of the bisubstrate analog was straightforward and took advantage of an intermediate (compound 2 in Figure 3.2) in the synthesis of a multisubstrate analog of biotin carboxylase (Amspacher et al., 1999). Since primary sulfur atoms are strong nucleophiles and the electrophilic carbon of 2 is adjacent to a carbonyl, the substitution of the chloride with the sulfur of Coenzyme A was readily accomplished. The conditions for the reaction were derived from the synthesis of a bisubstrate analog of serotonin N-acetyltransferase (Khalil and Cole, 1998). The reaction was carried out in triethylammonium bicarbonate buffer, pH 8.5, at room temperature. The bisubstrate analog was purified by extraction and reverse phase HPLC. Spectroscopic analysis (1H, 13C, and 31P NMR, and mass spectrometry) verified the structure of 1. Since removal of the benzyl-protecting group from the valeric acid side chain of biotin results in a decreased yield and considering that biotin, in vivo, is attached to a 22 kD protein, the benzyl group was not removed from 1. It should be noted that the enzyme does not appear to catalyze the synthesis of 1. When carboxyltransferase, Coenzyme A, and 2 were combined, the rate of formation of 1 was the same as in the absence of carboxyltransferase.

The bisubstrate analog was found to inhibit carboxyltransferase activity. The activity of carboxyltransferase was measured in the reverse direction with malonyl-CoA and biocytin as substrates. The inhibition of carboxyltransferase by 1 was determined by varying malonyl-CoA at increasing fixed concentrations of inhibitor, where biocytin was
held constant at a subsaturating level (Figure 3.3A). The inhibition of
carboxyltransferase was also determined by varying biocytin at increasing fixed
concentrations of 1, where malonyl-CoA was held constant at a subsaturating level
(Figure 3.3B). With malonyl-CoA as the variable substrate, compound 1 exhibited
competitive inhibition (Figure 3.3A), and had a slope inhibition constant (K_{is}) of 23 ± 2
µM. When biocytin was the variable substrate, noncompetitive inhibition was observed
(Figure 3.3B). Although 1 has a modest K_{i}, the main goal for making the inhibitor was to
synthesize a molecule that increases the affinity of biotin for the enzyme. To this end, the
inhibitor binds 350 times tighter to the enzyme than biocytin (biocytin has a K_{m} of 8
mM), and as a result will be useful for structural and mechanistic studies of ACC. It
should be noted that 1 also inhibits the activity of ACC in soluble extracts of murine
adipocytes (Levert and Waldrop, unpublished results).

The competitive inhibition pattern with respect to malonyl-CoA and
noncompetitive inhibition pattern with respect to biocytin indicate an ordered addition of
substrates to carboxyltransferase, with malonyl-CoA binding first (Figure 3.4). These
inhibition patterns confirmed the initial velocity patterns determined for
carboxyltransferase, which were equilibrium ordered with malonyl-CoA binding before
biocytin (Blanchard and Waldrop, 1998). An equilibrium ordered kinetic mechanism
also indicates that the off rate constants for the substrates are greater than the forward rate
constant to form products. Therefore, the K_{m} values for malonyl-CoA and biocytin are
equal to or very close to the dissociation constants of the substrates for the enzyme.

Using the K_{m} values for the two substrates (malonyl-CoA: 0.1 mM and biocytin: 8 mM)
Figure 3.3  Inhibition of carboxyltransferase by increasing concentrations of 1 with respect to malonyl-CoA (A) and biocytin (B). When malonyl-CoA was the variable substrate biocytin was held constant at 6.0 mM, and when biocytin was the variable substrate malonyl-CoA was held constant at 0.1 mM. The points are the reciprocal of the experimental velocities and the lines are derived from the best fit of the data to Eq. [3.1] (A) or Eq. [3.2] (B). The data was determined to fit best to these equations based on the square root of the residual least squares, or SIGMA (i.e. the sum of squares of differences between the experimental velocity and the calculated velocity). The best fit is determined through the lowest SIGMA value (Cleland, 1979).
Figure 3.4 The kinetic mechanism of carboxyltransferase, where A corresponds to malonyl-CoA, B is biocytin, P is carboxybiocytin, Q is acetyl-CoA, and I is the bisubstrate analog inhibitor (1). The inhibitor contains properties of both substrates and as a result can only bind to free enzyme, forming the dead-end complex EI. In this reaction scheme carboxybiocytin is shown leaving the enzyme before acetyl-CoA, however the order of product dissociation has not been experimentally determined and is assumed to occur in the reverse order of substrate binding.
as dissociation constants, the product of the dissociation constants for the two substrates is greater than the slope inhibition constant (23 µM) for 1. This is consistent with the inhibitor causing a conformational change in the enzyme. If the bisubstrate analog did not result in a conformational change, then the binding energy of the inhibitor would simply be a combination of the binding energies of the two substrates, and the $K_{is}$ would equal the product of the dissociation constants for the two substrates.

In summary, the formation of a bisubstrate analog has increased the affinity of biotin for carboxyltransferase by almost 350-fold. This increase in affinity combined with the ease of synthesis means that 1 will be useful for structural studies including crystallographic and spectroscopic studies. Equally important, 1 will also be very useful as a test inhibitor for developing assays for high-throughput screening, which will be necessary to find new herbicides, antibiotics, and anti-obesity agents directed against ACC.

**Endnotes**

1Carboxyltransferase from *E. coli* is routinely assayed in the non-physiological direction because of the availability of a facile spectrophotometric continuous assay that couples the production of acetyl-CoA with the reduction of NAD\(^+\) by the combined reactions of citrate synthase and malate dehydrogenase (Blanchard and Waldrop, 1998; Guchhait *et al.*, 1975). Carboxyltransferase assays also utilize biocytin in place of biotin because biocytin gives maximal velocities three orders of magnitude higher than biotin (Blanchard and Waldrop, 1998). Biocytin is biotin with a lysine attached to the carboxyl group of the valeric acid side chain via an amide linkage with the ε-amino group.

**References**


CHAPTER 4

A BIOTIN ANALOG INHIBITS ACETYL-CoA CARBOXYLASE ACTIVITY AND ADIPOGENESIS*

Introduction

Obesity is characterized by an increase in the number and size of adipocytes (Flier, 1995). During the course of adipogenesis the activities of several lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and ATP citrate lyase are increased (Mackall et al., 1976). The up-regulation of these enzymes suggests they could be targets for anti-obesity agents. For example, it has been shown recently that mice treated with inhibitors of fatty acid synthase resulted in decreased food intake and weight loss (Loftus et al., 2000). The hypothesis that acetyl-CoA carboxylase could be a target for anti-obesity agents was strengthened by a recent study demonstrating that mice lacking the gene coding for the mitochondrial isoform of acetyl-CoA carboxylase lost weight despite eating more food (Abu-Elheiga et al., 2001). In this study, we have demonstrated a pharmacological regulation of acetyl-CoA carboxylase activity and inhibition of adipocyte differentiation in 3T3-L1 cells.

Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the following two-step mechanism:

(1) Enzyme-biotin + HCO_3^- + MgATP \rightleftharpoons Mg^{++} \text{ Enzyme-biotin-CO}_2 + ADP + P_i

(2) Enzyme-biotin-CO_2 + Acetyl CoA \rightleftharpoons Malonyl CoA + Enzyme-biotin

The first-half reaction is carried out by the biotin carboxylase component of acetyl-CoA carboxylase and involves the ATP-dependent carboxylation of biotin with bicarbonate serving as the source of CO₂. The carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA in the second half reaction, which is catalyzed by carboxyltransferase. For both half-reactions, biotin remains covalently linked to the enzyme through an amide bond to a specific lysine residue of the biotin carboxyl carrier protein, and is designated as enzyme-biotin in the reaction scheme. Mammalian acetyl-CoA carboxylase contains all three components on a single polypeptide (Tanabe et al., 1975).

We have recently synthesized a bisubstrate analog inhibitor of the carboxyltransferase component of bacterial acetyl-CoA carboxylase (Figure 4.1) (Levert and Waldrop, 2002). Since human acetyl-CoA carboxylase is now a target for anti-obesity drugs, the question arose as to whether the bisubstrate analog we synthesized would inhibit mammalian acetyl-CoA carboxylase and in turn reduce lipid accumulation. Unfortunately, the bisubstrate analog contains the nucleotide ADP and therefore is not permeable to the cell membrane. However, the precursor to the analog, a chloroacetylated biotin derivative (CABI) (Figure 4.1), is sufficiently hydrophobic to diffuse across the cell membrane. The results of this study clearly demonstrate that treatment of 3T3-L1 cells with CABI inhibits the activity of acetyl-CoA carboxylase. Moreover, we have shown that CABI treatment inhibits the adipocyte differentiation of 3T3-L1 cells by blocking the induction of PPARγ and other adipocyte transcription factors.
Figure 4.1 Structures of CABI and CABI-CoA.
Experimental Procedures

**Materials.** Dulbecco’s Modified Eagles Media (DMEM) was purchased from Life Technologies. Bovine and fetal bovine serum (FBS) were obtained from Sigma and Life Technologies, respectively. PPARγ was a mouse monoclonal antibody from Santa Cruz Biotechnology. STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs from Santa Cruz Biotechnology. Streptavidin linked to horseradish peroxidase (HRP) was from Pierce. HPLC was performed using a Waters HPLC system equipped with a Waters 996 photodiode array detector. Analytical HPLC used a Discovery C-18 column (15 cm x 4 mm, 5 µm) purchased from Supelco. All other reagents were from Sigma or Aldrich.

**Cell Culture.** Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in DMEM with 10% bovine serum. Medium was changed every 48 hours. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (MIX), 1 µM dexamethasone (DEX), and 1.7 µM insulin. After 48 hours this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium until utilized for experimentation. CABI was dissolved in DMSO and added to the cell culture media at a 1 to 1000 dilution. Vehicle additions were performed in every experiment.

**Synthesis of chloroacetylated biotin.** Synthesis of chloroacetylated biotin was as previously described (Amspacher et al., 1999).
Preparation of Whole Cell Extracts. Monolayers of 3T3-L1 adipocytes were rinsed with phosphate-buffered saline (PBS) and then harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Nonidet P-40, 1 µM PMSF, 1 µM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 µM leupeptin, and 2 mM sodium vanadate for Western blot analysis. Samples were extracted for 30 minutes on ice and centrifuged at 10,000g at 4°C for 15 minutes. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions. This procedure was modified to prepare extracts for enzymatic analysis. The non-denaturing buffer contained 150 mM KCl, instead of NaCl, and there was no Triton X-100, Nonidet P-40 or any protease or phosphatase inhibitors included in the buffer. The cell monolayers were scraped in this buffer and were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 10,000g for 5 minutes and the supernatants were saved as cytosolic extract, and used to assay acetyl-CoA carboxylase activity.

Gel Electrophoresis and Immunoblotting. Proteins were separated in 5%, 7.5%, or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (Laemmli, 1970) and transferred to nitrocellulose (Biorad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 hour at room temperature. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) or streptavidin linked to HRP and enhanced chemiluminescence (Pierce).
Enzyme Assays. The activity of acetyl-CoA carboxylase from 3T3-L1 cell lysates was determined using a fixed time assay. Assays were performed by measuring the loss in acetyl-CoA and/or the production of malonyl-CoA at 5 min intervals for 20 min, using reverse phase HPLC. The rate of conversion of acetyl-CoA to malonyl-CoA was found to be linear for 20 min, and velocities were calculated by linear regression analysis of the malonyl-CoA concentration with respect to time. The reaction mixture contained 50 mM Tris, pH 7.5, 6 µM Acetyl-CoA, 2 mM ATP, 7 mM KHCO₃, 8 mM MgCl₂, 1 mM DTT, and 1 mg/ml bovine serum albumin. Cell lysates were preincubated (30 min, 25 °C) with bovine serum albumin (2 mg/ml) and potassium citrate (10 mM). Reactions were initiated by transferring 50 µl of preincubated cell lysate to the reaction mixture (final volume 200 µl) and incubated for 5-20 min at 25 °C. Reactions were terminated by addition of 50 µl 10% perchloric acid. Following termination of the reaction the samples were centrifuged (3 min, 10,000g) and analyzed by HPLC. A mobile phase of 10 mM KH₂PO₄, pH 6.7 (A) and MeOH (B) was used. The flow rate was 1.0 ml/min and the gradient was as follows: hold at 100% A for 1 min followed by a linear gradient to 30% B over the next 5 min, then hold at 30% B for 5 min. Using this method the retention times were 7.5 and 9.0 min for malonyl-CoA and acetyl-CoA, respectively.

Results

To test the ability of CABI to reduce acetyl-CoA carboxylase activity, confluent 3T3-L1 preadipocytes were treated for 4 hours with 10 µM CABI and whole cell extracts were prepared and immediately used to measure acetyl-CoA carboxylase activity by
analytical reverse phase HPLC. As shown in Figure 4.2A, the activity of acetyl-CoA carboxylase from cells treated with CABI was 0.30 nmol malonyl-CoA/min-mg and cells treated with DMSO had an activity of 1.40 nmol/min-mg. Thus, treatment of preadipocytes with CABI resulted in a 79% reduction in acetyl-CoA carboxylase activity. These samples were also analyzed for the expression of acetyl-CoA carboxylase using streptavidin HRP (Figure 4.2B). These results clearly demonstrate that the reduced activity of acetyl-CoA carboxylase is not due to altered expression levels of the enzyme.

Since treatment of 3T3-L1 preadipocytes with CABI reduced acetyl-CoA carboxylase activity, we examined the effects of CABI on the adipogenesis of these cells. At two days post-confluence, the 3T3-L1 cells were exposed to the normal differentiation cocktail (FBS, MIX, DEX, and insulin) for 48 hours in the presence or absence of various doses of CABI. After 48 hours, the cells were maintained in 10% FBS in DMEM. A vehicle addition of DMSO was also performed. CABI or DMSO was added to the cells in a 1 to 1000 dilution into the cell culture medium every 24 hours. The isolation of whole cell extracts and Oil Red O staining was performed one week following treatment with the induction cocktail. Adipogenesis was assessed by examining the expression of several transcription factors, PPARγ and STATs 1 and 5A, which are highly induced during adipogenesis (Rosen et al., 2000) and by examining lipid accumulation, as judged by Oil Red O staining. Optimal differentiation, as judged by PPARγ expression and Oil red O staining, was achieved when 3T3-L1 cells were exposed to the normal differentiation cocktail containing FBS, MIX, DEX, and insulin, which resulted in 100%
Figure 4.2 CABI treatment of 3T3-L1 preadipocytes results in decreased acetyl-CoA carboxylase activity, but not protein levels, in isolated cell extracts. (A) Whole cell extracts were prepared from 3T3-L1 preadipocytes following a 4 hour treatment with either DMSO or 10 µM CABI dissolved in DMSO. Acetyl-CoA carboxylase activity was measured by analytical reverse phase HPLC. (B) The whole cell extracts used to measure acetyl-CoA carboxylase activity were used to examine acetyl-CoA carboxylase protein levels. One hundred µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and incubated for 4 hours with 2 µg/ml streptavidin HRP. Results were visualized by detecting HRP with enhanced chemiluminescence (Pierce).
adipocyte conversion as previously reported (Yeh et al., 1995). However, we observed a
dose-dependent inhibition of differentiation in the presence of CABI. Exposure of
differentiating adipocytes to 17 µM or 8 µM CABI blocked the induction of
PPARγ expression (Figure 4.3) and lipid accumulation as judged by Oil Red O staining
(Figure 4.4). Parallel with the 100% conversion, STATs 1 and 5A were highly
expressed, and the induction in expression of these two transcription factors was also
inhibited in a dose-dependent manner by CABI treatment. The specificity of this
treatment is demonstrated by examining the expression of STAT 3, a protein whose
expression is not substantially regulated during differentiation. As shown in Figure 4.3,
the expression of STAT 3 was unaffected by CABI treatment and is shown as a loading
control.

Discussion

The novel observations in this study include the ability of CABI to diffuse into
3T3-L1 cells and reduce the activity of cellular acetyl-CoA carboxylase and to inhibit the
adipogenesis of 3T3-L1 cells in a dose dependent manner by blocking the induction of
PPARγ expression. To account for these observations we suggest that upon entering the
cell, CABI reacts with endogenous Coenzyme A to form the bisubstrate analog CABI-
CoA. Once formed the bisubstrate analog inhibits the cytosolic and/or mitochondrial
isoforms of acetyl-CoA carboxylase. This hypothesis is supported by the observations
that CABI had no effect on acetyl-CoA carboxylase activity in isolated cellular extracts
(data not shown). However, the bisubstrate analog CABI-CoA was indeed able to inhibit
acetyl-CoA carboxylase activity when added to isolated cellular extracts in vitro.
Figure 4.3 CABI blocks the induction of adipogenic transcription factors in a dose dependent manner. Whole cell extracts were prepared one week after the induction of differentiation in the presence of various doses of CABI, as indicated at the top of the figure. For each condition, the cells were cultured in the presence or absence of CABI or DMSO, and the cells were treated every 24 hours. One hundred µg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The molecular mass of each protein is indicated to the left of the blot in kilodaltons. The detection system was horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce). This is a representative experiment independently performed three times.
Figure 4.4  CABI inhibits lipid accumulation in differentiating 3T3-L1 cells. Oil Red O staining was performed on 3T3-L1 cells one week following the induction of differentiation. Cells were induced to differentiate at 2 days post-confluence and CABI was added at the initiation of differentiation. After 48 hours, the induction medium was replaced with DMEM. The cells were treated every 24 hours with a fresh bolus of CABI or DMSO. Each plate was treated with the following concentrations of CABI: (A) 0 µM, DMSO control, (B) 4 µM, (C) 8 µM, (D) 17 µM. This is a representative experiment independently performed three times.
Moreover, there is precedent for enzyme inhibitors forming intracellularly by reaction of a precursor with a metabolite. Examples include finasteride, which inhibits 5α reductase (Bull et al., 1996), isoniazid, which inhibits the mycobacterial enzyme InhA (Rozwarski et al., 1998), and a bisubstrate analog inhibitor of serotonin N-acetyltransferase (Khalil et al., 1999). The first two examples are used clinically to treat benign prostatic hypertrophy and tuberculosis, respectively. Our attempts to detect CABI-CoA in extracts from CABI treated 3T3-L1 cells have been unsuccessful using HPLC with absorption optics. However, studies are underway to detect the bisubstrate analog CABI-CoA in cellular extracts with more sensitive methods.

The observation that the expression of STAT3 was unaffected by CABI is very important because it suggests that CABI is not acting as a non-specific alkylating agent. Moreover, the fact that the level of acetyl-CoA carboxylase did not decrease with CABI treatment further indicates that CABI is not exerting a general toxic effect. It should be noted that the inhibition of differentiation by CABI was reversible. If the addition of CABI was not repeated every 24 hours, the cells started to differentiate.

We have recently determined the inhibition constant of the bisubstrate analog, CABI-CoA, for bacterial acetyl-CoA carboxylase is 23 µM (Levert and Waldrop, 2002). If the inhibition constant for the murine acetyl-CoA carboxylase in 3T3-L1 cells is similar to the bacterial enzyme, then it is tempting to speculate how an inhibitor with such a modest $K_i$ can result in such a significant biological effect. The answer begins by noting the importance of acetyl-CoA carboxylase for cell growth and the recent demonstration that a gene knockout of cytosolic acetyl-CoA carboxylase is embryonic
lethal (Abu-Elheiga et al., 2001). Our goal was not to abolish the activity of all cellular acetyl-CoA carboxylase and induce cell death. Instead, the objective was to attenuate the activity of acetyl-CoA carboxylase and prevent lipid accumulation. To this end, a molecule with a 23 µM $K_i$ value would serve this purpose, while a molecule with a nM or lower $K_i$ value would have greater cytotoxicity.

In summary, the results presented in this paper are the first demonstration of a link between a pharmacological modulation of cytosolic acetyl-CoA carboxylase and inhibition of adipogenesis. These studies support the gene knockout experiments in mice, which indicate that acetyl-CoA carboxylase is a very promising target for anti-obesity agents (Abu-Elheiga et al., 2001). It will be interesting to examine the effects of CABI on other types of cells in which acetyl-CoA carboxylase is up-regulated such as breast cancer cells (Thupari et al., 2001). We are currently searching for alternative solvents for CABI and trying to synthesize a more soluble precursor so that these compounds can be tested in rodents.

References


CHAPTER 5
CONCLUSIONS

This dissertation describes three separate studies of acetyl-CoA carboxylase. The first study was a direct test of the proposed hypothesis for the deprotonation of biotin by biotin carboxylase. Site-directed mutagenesis studies of the biotin carboxylase subunit of acetyl-CoA carboxylase have demonstrated that the active-site residues cysteine 230 and lysine 238 do not act as acid-base catalysts to deprotonate biotin. The results from this study suggest that Cys230 is involved in the binding of ATP, and plays no role in catalysis. If Cys230 acted as a catalytic base to remove the 1’-N proton from biotin, then removal of this residue should have had a significant effect on the ability of the enzyme to produce carboxybiotin. However, the C230A mutant retained the ability to catalyze the production of carboxybiotin, and did so at a rate almost equivalent to the wild-type enzyme. The results of the bicarbonate-dependent ATPase assay also suggest that Lys238 is involved in binding to ATP. This is consistent with the results of the affinity labeling of biotin carboxylase with adenosine diphosphopyridoxal, which also suggested Lys238 interacts with the γ-phosphate of ATP (Kazuta et al., 1998).

Unlike the C230A mutant, which had little effect on the biotin-dependent ATPase activity of biotin carboxylase, the K238Q mutant had a significant effect on this reaction. Although the K238Q mutant retained the ability to hydrolyze ATP in the presence of bicarbonate at a rate equivalent to wild-type enzyme, there was no increase in the rate of ATP hydrolysis in the presence of biotin. This mutation also abolished the enzymes
ability to catalyze the formation of carboxybiotin. This suggests that lysine 238 is either involved in the enzymatic production of carboxybiotin, or this mutation has prevented biotin from binding to the enzyme. The stimulation by biotin of the ATP synthesis reaction observed with the K238Q mutant demonstrates that biotin can still bind to the enzyme, although with a reduced affinity. Thus, the question arises as to whether lysine 238 could act as a catalytic base to deprotonate biotin. However, the inactivation studies using N-ethylmaleimide demonstrated that Lys238 has a pK of at least 9.4. This high pK value suggests Lys238 is protonated at the onset of the reaction, and cannot act as a catalytic base.

If Cys230 and Lys238 do not act in concert to remove the 1’-N proton from biotin, then how is biotin deprotonated? With the current lack of evidence to support the existence of an active site residue acting as a catalytic base, an alternative mechanism has been proposed to account for biotin deprotonation. The alternative hypothesis relies on the observation that transfer of the carboxyl group from carboxyphosphate to biotin requires one of the phosphate oxygens of carboxyphosphate to be protonated. Thus, one of the phosphate oxygens may act as a catalytic base and remove the proton from biotin. Similar substrate-assisted mechanisms for enzyme catalysis have also been proposed for aspartate transcarbamylase (Gouaux et al., 1987) and the GTPase activity of the protein ras (Schweins et al., 1995). This alternative mechanism also suggests a possible role for Lys238. The results of this study found that Lys238 is involved in binding ATP and in the transfer of the carboxyl group from carboxyphosphate to biotin. If Lys238 binds the γ-phosphate of ATP and in turn the phosphate group of carboxyphosphate, then Lys238
may help to orient the phosphate group for proton extraction from the 1’-N atom of biotin (Figure 1). This is analogous to a study of D-Ala:D-Ala ligase where the lysine residue equivalent to Lys238 was mutated and the researchers concluded that the protonated ε-amino group interacted with the γ-phosphate of ATP and helped to orient the γ-phosphate for transfer to D-alanine (Shi and Walsh, 1995).

![Proposed reaction mechanism for biotin carboxylase.](image)

**Figure 5.1** Proposed reaction mechanism for biotin carboxylase. The phosphate group of carboxyphosphate acts as the catalytic base to deprotonate biotin while lysine 238 helps to orient the phosphate group for proton extraction. Although the 1’-N atom of biotin has a pK of 17.4 (Fry *et al.*, 1985) and the pK of the phosphate oxygen is 12.4, this mechanism could occur through the stabilization of the enol form of biotin through an interaction with a basic residue and the carbonyl oxygen of biotin.

This dissertation also demonstrates that a bisubstrate analog of the carboxyltransferase component of acetyl-CoA carboxylase can be synthesized by covalently linking coenzyme A with the 1’-N atom of biotin via an acyl bridge. This bisubstrate analog was found to inhibit the activity of carboxyltransferase, and
demonstrated competitive inhibition versus malonyl-CoA and noncompetitive inhibition versus biocytin. This pattern of inhibition indicates an ordered addition of substrates to the enzyme, with malonyl-CoA binding first. These inhibition patterns confirmed the initial velocity patterns determined for carboxyltransferase, which were equilibrium ordered with malonyl-CoA binding before biocytin (Blanchard et al., 1998).

The bisubstrate analog inhibited the activity of carboxyltransferase with a binding constant of $23 \pm 2 \, \mu M$. Thus, the inhibitor binds to the enzyme 350 times tighter than biotin. This suggests the inhibitor can be useful for structural studies of carboxyltransferase. This bisubstrate analog will also be useful in the search for new antibiotics, herbicides, and anti-obesity agents. In fact, Pfizer Inc. (New York City, New York, 235 E. 42^{nd} Street) has used this inhibitor to help develop an assay for high-throughput screening. The bisubstrate analog inhibitor of carboxyltransferase was used to validate their assay, and aid in the search for new antibiotics.

Since acetyl-CoA carboxylase plays an essential role in the synthesis of fatty acids, the bisubstrate analog could serve as an anti-lipogenic agent in cells treated with the inhibitor. Since coenzyme A contains a nucleotide it is not permeable to the cell membrane. However, the chloroacylated biotin analog is sufficiently hydrophobic and will pass through the cell membrane. Once inside the cell the chloroacylated biotin could react with endogenous coenzyme A to form the bisubstrate analog, and inhibit the activity of acetyl-CoA carboxylase. A murine 3T3-L1 cell line was used to examine the effects of the chloroacylated biotin analog on adipocyte differentiation. Under the conditions of cell culture, the 3T3-L1 cell line will differentiate from preadipocytes into cells with the
morphological and biochemical properties of adipocytes (Green and Kehinde, 1974; Green and Kehinde, 1976). These cells demonstrated a dose-dependent inhibition of differentiation upon exposure to the chloroacylated biotin analog. Treatment with chloroacylated biotin blocked the induction of the transcription factors that normally occurs with adipogenesis and also prevented lipid accumulation. The treated cells also demonstrated a significant decrease in the activity of acetyl-CoA carboxylase, although the enzyme levels remained constant. Since the chloroacylated biotin analog does not inhibit the activity of acetyl-CoA carboxylase it is likely that the bisubstrate analog was formed intracellularly, which would result in decreased acetyl-CoA carboxylase activity. This supports the recent studies that suggest acetyl-CoA carboxylase is a target for anti-obesity therapeutics.

References


Matrix assisted laser desorption ionization mass spectrometry analysis of the bisubstrate analog inhibitor.
$^1$H-NMR spectrum of the bisubstrate analog inhibitor (400 MHz)
$^{13}$C-NMR spectrum of the bisubstrate analog inhibitor (500 MHz)
$^{31}$P-NMR spectrum of the bisubstrate analog inhibitor (400 MHz)
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VITA

Keith Logan Levert was born on May 8, 1968, in New Orleans, Louisiana. At the age of four he moved to Baton Rouge, Louisiana, where he has remained since. Keith received his Bachelor of Science degree from Louisiana State University in May, 1997. In August, 1997, he entered the graduate program at Louisiana State University in the Department of Biological Sciences. As a graduate student Keith worked in the laboratory of Dr. Grover L. Waldrop, where he worked on the enzyme acetyl-CoA carboxylase. After graduation in May, 2002, Keith plans to join the research group of Dr. Brian Eckenrode where he will study forensic science.