

**SITE-DIRECTED MUTAGENESIS STUDIES OF *E. COLI* BIOTIN CARBOXYLASE**

A Dissertation

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

in

The Department of Biological Sciences

by  
Valerie M. Sloane  
B.S., University of Louisiana at Lafayette, 1995  
May 2004

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Grover Waldrop, my major professor, for his invaluable guidance and helpful insights throughout this project, as well as for obtaining grants for the research. I am also indebted to Professor Simon Chang, Associate Professor Patrick DiMario, Associate Professor Jacqueline Stephens, Assistant Professor Anne Grove, and Assistant Professor Britt Thomas for serving on my committee and for a thoughtful reading of each chapter. Dr. Carol Blanchard and Tee Bordelon especially deserve my thanks for their careful tutelage in molecular biology and protein purification techniques. Finally, I wish to acknowledge Karen Broussard, my mother, Francis Broussard, and Craig Moses for their unwavering confidence in me. I would not have achieved this task without their vital encouragement.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	ii
ABSTRACT.....	iv
CHAPTER 1. INTRODUCTION .....	1
The Biotin-Dependent Enzymes .....	1
Acetyl-CoA Carboxylase .....	3
Regulatory Aspects of Acetyl-CoA Carboxylase .....	4
<i>E. coli</i> Acetyl-CoA Carboxylase – A Model System .....	7
Objectives and Rationale for Study .....	15
References.....	16
CHAPTER 2. SITE-DIRECTED MUTAGENESIS OF ATP-BINDING RESIDUES OF <i>E. COLI</i> BIOTIN CARBOXYLASE: INSIGHT INTO THE MECHANISM OF CATALYSIS.....	19
Introduction.....	19
Materials and Methods.....	22
Results .....	25
Discussion.....	32
References.....	40
CHAPTER 3. KINETIC CHARACTERIZATION OF MUTATIONS FOUND IN PROPIONIC ACIDEMIA AND METHYLCROTONYLGLYCINURIA: EVIDENCE FOR NEGATIVE COOPERATIVITY IN BIOTIN CARBOXYLASE.....	43
Introduction.....	43
Materials and Methods.....	46
Results.....	50
Discussion.....	62
References.....	67
CHAPTER 4. CONCLUSIONS .....	69
APPENDIX: LETTER OF PERMISSION.....	73
VITA.....	74

## ABSTRACT

Acetyl-CoA carboxylase is an essential enzyme in all plants, animals and bacteria, where it catalyzes the committed step in fatty acid synthesis. The *Escherichia coli* form of the enzyme has three functional components that dissociate readily: biotin carboxylase, carboxyltransferase, and a biotin carboxyl carrier protein. The biotin carboxylase component catalyzes the ATP-dependent carboxylation of biotin, using bicarbonate as the carboxylate source. Biotin carboxylase is a member of a functionally diverse superfamily of proteins known as the ATP-grasp enzymes. In the first study, four residues of biotin carboxylase, Lys 116, Lys 159, His 209 and Glu 276, were selected for site-directed mutagenesis based on their structural homology and strict conservation among the ATP-grasp enzymes. The resulting mutants were subject to kinetic characterization. The  $K_m$  for ATP for all four mutants was significantly elevated relative to the wild type enzyme, implicating the residues in binding ATP. The  $V_{max}$  for the biotin-dependent ATPase reaction was 30- to 260-fold lower than wild type, suggesting that the mutations have misaligned the substrates for optimal catalysis.

In the second study, three more biotin carboxylase mutants were made based on their homology to naturally occurring mutations of human biotin-dependent carboxylases. The three mutations result in metabolic diseases, indicating that the residues involved are functionally important. The mutants M169K, R338Q, and R338S were constructed and characterized. The two Arg mutants displayed uncoupling of biotin carboxylation from ATP hydrolysis, suggesting that the residue Arg 338 is important for aligning the carboxyphosphate intermediate for optimal carboxyl transfer to biotin. Interestingly, all three mutants displayed negative cooperativity with respect to bicarbonate, suggesting a communication between the two subunits of biotin

carboxylase. Additionally, the level of residual biotin-dependent ATPase activity for M169K and R338S was consistent with the severity of the phenotypes of the patients carrying the corresponding mutations, thus establishing a molecular basis for the diseases.

# CHAPTER 1

## INTRODUCTION

### The Biotin-Dependent Enzymes

Biotin, or vitamin H, is an indispensable cofactor for a small number of enzymes that catalyze the transfer of carboxyl groups. These enzymes participate in a variety of metabolic functions, from gluconeogenesis to urea degradation to sodium transport. The biotin prosthetic group, depicted in Figure 1.1, contains a ureido and a tetrahydrothiophene ring fused together with a valeric acid side chain attached at the 2 position of the tetrahydrothiophene. The cofactor is attached to the enzyme via an amide linkage between the valeric side chain and the  $\epsilon$ -amino group of a specific, highly conserved lysine residue. Biotin can be carboxylated at the 1' nitrogen, thereby serving as the  $\text{CO}_2$  carrier for the enzyme.

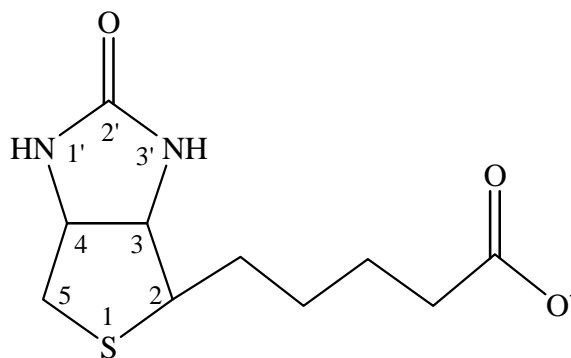
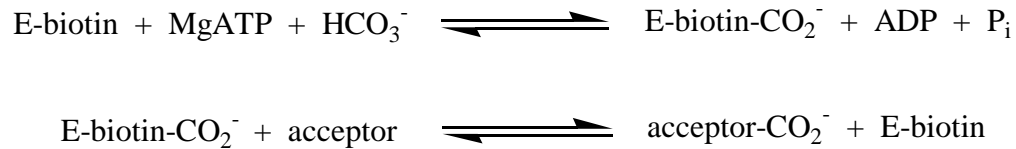


Figure 1.1

All of the reactions catalyzed by the biotin-dependent enzymes occur in two steps: the first, in which biotin is carboxylated, and the second, in which the carboxyl group is relinquished to an acceptor molecule. The enzymes are further categorized according to whether they catalyze the fixation of  $\text{CO}_2$  (Class I), decarboxylation, releasing the  $\text{CO}_2$  as bicarbonate (Class II), or the transfer of a carboxyl group from one molecule to another (Class III).

Carboxylases (Class I). There are six Class I carboxylases (also called biotin-dependent carboxylases), all of which use bicarbonate as the carboxylate source. The general reaction catalyzed by the Class I enzymes is indicated in Scheme 1.1. The fixation of carbon requires hydrolysis of one molecule of ATP, followed by the transfer of the carboxyl group to an acceptor molecule.

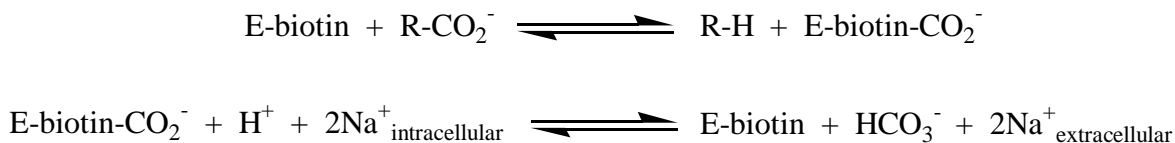


Scheme 1.1

The biotin cofactor, covalently bound to the enzyme, is indicated as E-biotin. The six biotin-dependent carboxylases include pyruvate carboxylase, propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, acetyl-CoA carboxylase, geranyl-CoA carboxylase, and urea carboxylase. The first four biotin-dependent carboxylases are present in animals, where they play important roles in gluconeogenesis, amino acid catabolism, and fatty acid biosynthesis, respectively. Urea carboxylase has been characterized in yeast (1, 2), *Chlamydomonas*, and *Chlorella* (3), where it allows the organism to utilize urea as a nitrogen source by degrading urea to ammonia. Geranyl-CoA carboxylase, an enzyme previously thought to exist only in bacterial systems, has recently been identified in plants (4), where it plays a role in isoprenoid catabolism.

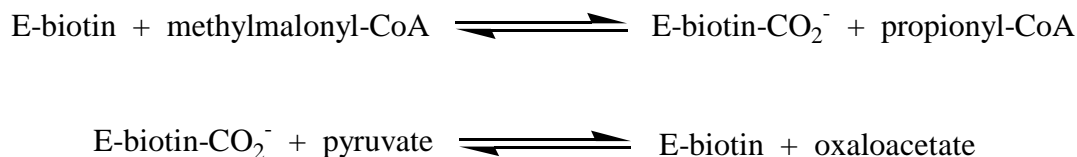
Decarboxylases (Class II). The decarboxylases of Class II include oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, and glutaconyl-CoA decarboxylase. The general reaction is indicated in Scheme 1.2. Note that the decarboxylases do not transfer the carbon to an acceptor, but rather release it as bicarbonate. Generally, these enzymes are found in the membranes of obligately anaerobic bacteria, where they harness the free energy of

decarboxylation to transport sodium ions into the periplasm. The resulting electrochemical gradient is then used to synthesize ATP (5).



Scheme 1.2

Transcarboxylase (Class III). There is only one known transcarboxylase, which catalyzes a carboxyl transfer from methylmalonyl-CoA to pyruvate. Transcarboxylase was discovered in the bacterium *Propionibacterium shermanii* and is involved in the synthesis of propionic acid from pyruvic acid (6). The two step reaction is indicated in Scheme 1.3.



Scheme 1.3

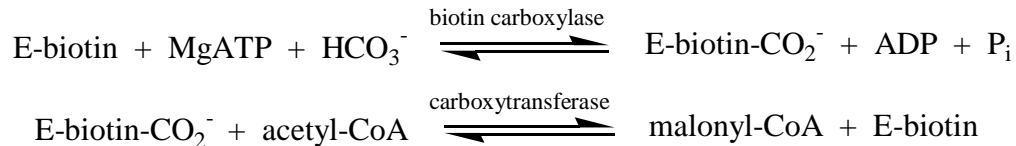
Covalent Attachment of Biotin. The isolation of biocytin ( $\epsilon$ -N-biotinyl-L-lysine) from yeast extract was the first evidence that the attachment site on a biotin-dependent enzyme was a lysine residue (7). This posttranslational modification is carried out by holocarboxylase synthase (HCS), also known as biotin protein ligase (BPL). The site of covalent attachment contains the nearly invariant tetrapeptide sequence Ala-Met-Lys-Met (8). Although it was once thought that this region served as a recognition site for HCS, mutagenesis studies have indicated that the methionines play a role in the carboxylation of biotin (9, 10).

### **Acetyl-CoA Carboxylase**

The focus of this dissertation concerns the Class I enzyme acetyl-CoA carboxylase (ACC), an essential enzyme found in all plants, animals, and bacteria (but absent from Archaea).

ACC catalyzes the first and committed step in the *de novo* synthesis of long-chain fatty acids. The enzyme was first discovered in 1958 when Wakil *et al.* observed that avidin, a protein that irreversibly binds biotin, was inhibitory toward the carboxylation of acetyl-CoA (11).

The overall reaction catalyzed by ACC converts acetyl-CoA to malonyl-CoA in the following two-step scheme:



Scheme 1.4

The two half-reactions occur at separate active sites on the enzyme. The first half-reaction is catalyzed by the homodimeric biotin carboxylase component, while the second is catalyzed by the heterodimeric carboxyltransferase component. The small biotin carboxyl carrier protein (BCCP) contains the conserved lysine residue to which biotin is covalently attached. In bacterial ACC, the two enzymatic components exist as separate proteins, whereas in eukaryotes, presumably as a result of gene fusion, the functions are on a single multifunctional polypeptide chain. A few bacterial species, such as *Mycobacterium tuberculosis* (12) and *Myxococcus xanthus* (13), have the BCCP and biotin carboxylase components on the same polypeptide chain. However, in the majority of bacterial systems, the BCCP is a separate subunit.

### Regulatory Aspects of Acetyl-CoA Carboxylase

The essential activity of ACC is tightly regulated. The malonyl-CoA derived from ACC is a substrate for the second enzyme in the fatty acid biosynthetic pathway: fatty acid synthase (FAS). This enzyme is a multifunctional complex which, like ACC, is also found in all plants, animals, and bacteria, including some Archaea. The acyl carrier protein (ACP) is the central component of the complex which anchors the growing fatty acyl chain until it is released as

palmitate (C16). Feedback inhibition by fatty acid synthase intermediates is an important mechanism of ACC control (14, 15), however, a number of other regulatory mechanisms are known.

Regulation in Prokaryotes. Davis and Cronan recently observed that *E. coli* ACC is partially inhibited by acylated derivatives of ACP (16). Interestingly, the acyl chain length carried by ACP was equally inhibitory between C6 and C20. Bacterial ACC is also regulated at the transcriptional level. The transcription of *E. coli* ACC genes was shown to be closely related to the rate of cell growth, which would be expected since fatty acids in bacteria are required only for the synthesis of membrane lipids (17). Although the mechanism of transcriptional activation of ACC is as yet unclear, James and Cronan recently reported that BCCP may have an autoregulatory function ( \*18 ). The *accB* and *accC* genes of *E. coli*, which encode BCCP and biotin carboxylase, respectively, are cotranscribed as an operon (referred to as the *accBC* operon). The carrier protein was found to inhibit transcription of *accBC*, suggesting a possible feedback mechanism of transcriptional control. The unlinked *accA* and *accD* genes, which encode the two subunits of carboxyltransferase, appear to be under a different set of transcriptional controls (17, \*18).

Regulation in Eukaryotes. Eukaryotic ACC has a more complex system of regulation that includes assembly of the enzyme into active multimers or disassembly into inactive protomers. Yeast ACC forms a tetramer in its active state and is regulated by a phosphorylation/dephosphorylation cycle in which phosphorylation favors the inactive protomer. The SNF1 gene in yeast encodes a 72 kDa protein kinase that has been shown to phosphorylate ACC *in vitro* (19). An *in vivo* study (20) indicated that the SNF1 kinase causes inhibition of ACC in yeast cells, and that the inhibition was due to a covalent modification (presumably

phosphorylation). In animal tissues, active ACC polymerizes into long filaments (14), a state which is activated by the Krebs cycle intermediates citrate and isocitrate. In this way, high levels of citrate can signal a metabolic switch from fuel oxidation to fuel storage. On the other hand, fatty acyl-CoA's inhibit animal ACC, favoring the protomer state. Palmitoyl-CoA, stearoyl-CoA, and arachidyl-CoA are the most effective acyl-CoA inactivators of ACC (21).

Phosphorylation/dephosphorylation of ACC is also known to be a regulatory mechanism in animal systems. Glucagon and epinephrine stimulate the phosphorylation of ACC, which triggers disassembly of the filaments into inactive monomers, while insulin stimulates dephosphorylation, reversing the inhibition (14).

Biomedical Relevance of Mammalian ACC Regulation. Two distinct isozymes of ACC were discovered in human tissues: ACCA, which is cytoplasmic, and ACCB, which is localized to the mitochondria. (22, 23). Interestingly, the expression of both isoforms of ACC, as well as FAS, are upregulated in *in situ* breast carcinoma cells (tumor cells in a preinvasive stage of progression) (24). Although the relationship between breast cancer and increased fatty acid production is not very clear, increased flux through the fatty acid synthesis pathway could presumably increase the production of certain signal-transducing lipids. A recently characterized gene called BRCA1 encodes a 220-kDa protein believed to act as a tumor suppressor. The BRCA1 protein has been shown to interact with numerous proteins involved in DNA damage repair, cell cycle regulation, and tumor suppression (\*25 ). In addition, Magnard *et al.* demonstrated that the BRCA1 protein interacts specifically with ACCA, and that tumor-inducing mutations in BRCA1 can disrupt this interaction (26). Thus, the ACCA/BRCA1 interaction can be an indicator of tumor predisposition, and ACC is a potential target for anti-cancer therapeutics.

The isozymes of ACC have homologs in mice. The fact that there are two isozymes presents the opportunity to study the suppression of ACC activity through gene knockout experiments. A noteworthy study by Wakil *et al.* involving ACC2<sup>-/-</sup> mice demonstrated that the mutant mice had lower body fat and decreased storage of glycogen and lipid droplets in the liver compared to wild type mice, despite consuming 20-30% more food than wild type (27). This was postulated to be a result of uncontrolled fatty acid oxidation due to decreased levels of malonyl-CoA (malonyl-CoA is a negative regulator of fatty acid oxidation). However, with respect to growth rate, morphology, and breeding, the ACC2-deficient mice were normal. The ACC2 isozyme is thus a possible target for anti-obesity therapies as well if it can be inhibited selectively over the ACC1 isozyme.

Recently, it was observed that leptin, a peptide hormone expressed in adipocytes, suppresses the activity of ACC through the activation of a 5'-AMP-activated protein kinase (28). Leptin secretion is associated with increased cellular energy expenditure and decreased hunger, and because it is upregulated as adipocytes increase in size, it serves as a barometer for the cell's nutritional state. In fact, the increased feeding in the ACC2-deficient mice was attributed to a decrease in leptin content in the plasma (27).

### ***E. coli* Acetyl-CoA Carboxylase – A Model System**

A large portion of the current knowledge on the mechanism of biotin-dependent carboxylases has derived from *E. coli* ACC. This enzyme provides an ideal model system for enzymological study, primarily due to the fact that its three separate subunits are easily resolved, and the two enzymatic components, biotin carboxylase and carboxytransferase, have been cloned and overexpressed. Moreover, both subunits will use free biotin as a substrate, thereby simplifying kinetic analyses.

Perhaps the most valuable and informative aspect of the *E. coli* ACC model system is the availability of structural information. In 1994, the crystal structure of the biotin carboxylase subunit was solved to 2.4 Å resolution (29). The structure was later refined to 1.9 Å resolution when the enzyme was cocrystallized with ATP, allowing many interactions between the substrate and the active site residues to be discerned (30).

This dissertation will focus on the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase. The studies described herein take advantage of the wealth of structural information on *E. coli* biotin carboxylase as well as the facile purification of the overexpressed protein.

Structural Aspects of *E. coli* Biotin Carboxylase. The original crystal structure of biotin carboxylase revealed that the 449-amino acid protein contains three distinct structural domains. The N-terminal domain has a dinucleotide binding motif with five parallel beta-strands. The small central domain (called the “B-domain”), which protrudes conspicuously from the rest of the enzyme, consists of two alpha-helices and three beta-strands which are attached to the protein by a glycine-rich region. This region was not well defined in the crystal structure, suggesting it may be flexible. In fact, the B-domain was postulated to close down over the active site upon the binding of substrate. The C-terminal domain contains two antiparallel beta-sheets: one eight-stranded and a smaller three-stranded sheet. There are also seven alpha helices in this domain. The residues Arg 338, His 209, and Glu 276 were suggested to play important catalytic roles based on their location in the active site pocket (29).

Soon after the the crystal structure of biotin carboxylase was solved, Artymiuk, *et al.* noticed that biotin carboxylase had a strong structural homology with two other metabolically unrelated enzymes: D-ala-D-ala ligase and glutathione synthetase. Artymiuk *et al.* (31) used a program to compare the 3D coordinates of biotin carboxylase to those of other proteins cataloged

in the Protein Data Bank. The folds of the three proteins were so strikingly similar that an evolutionary relationship seemed certain. Interestingly, they share a sequence identity of only 11% (31), suggesting that evolution can preserve useful protein folds without conserving the sequence. After the development of the PSI-BLAST (Position-Specific Iterative BLAST) search, many more proteins with similar folds to that of biotin carboxylase were discovered and included in a superfamily called the ATP-grasp enzymes (32). Included in the superfamily are carbamyl phosphate synthetase, glycylamide ribonucleotide transformylase, and of course, all biotin-dependent carboxylases. Not surprisingly, the ATP-grasp enzymes all catalyze reactions with a similar chemistry, namely, the ATP-dependent ligation of a carboxylate-containing substrate to an amine- or thiol-containing substrate. In addition, the ATP-grasp enzymes are thought to activate their carboxyl substrates via the formation of acylphosphate intermediates (32).

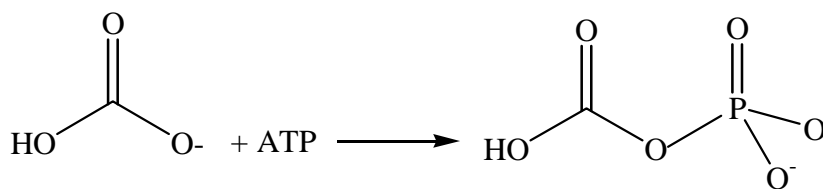
The subsequent crystal structure of biotin carboxylase with bound nucleotide revealed that the B-domain does indeed close to cover the active site upon ATP binding (30). This discovery is in good agreement with the structure of carbamyl phosphate synthetase, which was cocrystallized with the nucleotide analog AMPPNP and revealed a similar closure (33). Additionally, the closed conformation of the ATP-bound structure of biotin carboxylase brought a number of residues into the active site pocket that had not been predicted from the unliganded structure. Among these residues were Lys 116, Lys 159, and Met 169 (30). Moreover, sequence alignments of ATP-grasp enzymes revealed that these three residues were highly conserved throughout the superfamily (32).

Mechanistic Aspects of *E. coli* Biotin Carboxylase. Mechanistically, biotin carboxylase (as well as the other biotin-dependent carboxylases) faces two problems with respect to carboxyl

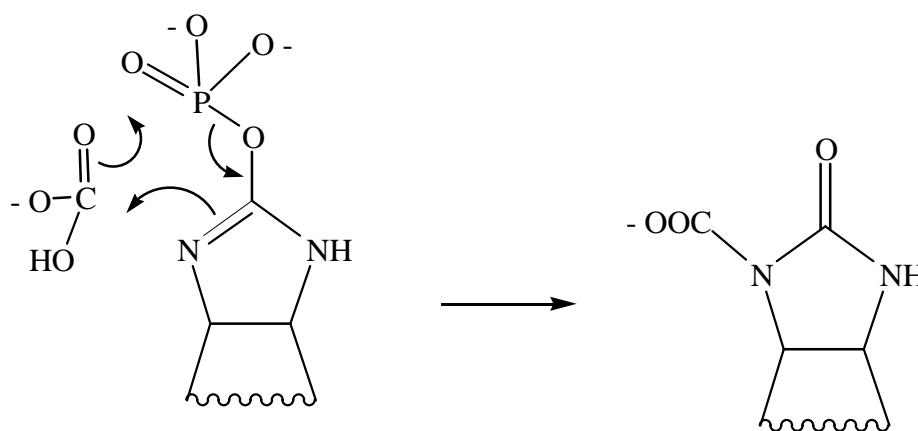
transfer to biotin: (1) bicarbonate is a poor electrophile and (2) the 1' nitrogen of biotin is a poor nucleophile. How can these two reactants be activated so that this chemistry is feasible? These aspects of the catalytic mechanism have been extensively studied.

Activation of Bicarbonate. The currently accepted mechanism for activation of bicarbonate is the formation of a carboxyphosphate intermediate, as shown in Scheme 1.5. Carboxyphosphate is a highly unstable entity with an estimated half-life of 70 msec (34).

Since carboxyphosphate has never been isolated per se, a number of alternative mechanisms have been proposed which did not involve the formation of carboxyphosphate. One of the more well-known schemes was proposed by Calvin and Pon (35) and Lynen (36), in which ATP reacts directly with biotin to form *O*-phosphobiotin. This intermediate then reacts with bicarbonate to give *N*-1 carboxybiotin (Scheme 1.6).



Scheme 1.5

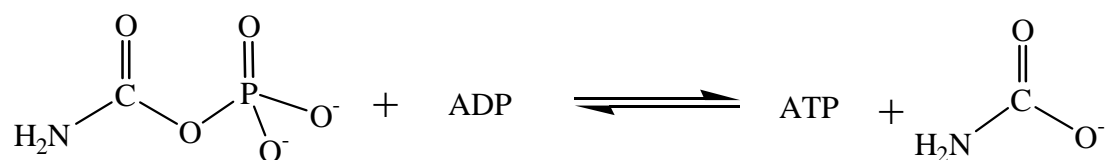


Scheme 1.6

Evidence in support of the intermediacy of carboxyphosphate and against that of *O*-phosphobiotin derives from carbamyl phosphate synthetase because of its mechanistic homology to the biotin-dependent carboxylases. Although this enzyme does not use the cofactor biotin, it does catalyze an ATP-dependent carboxylation reaction for which the carbon source is bicarbonate. Using a diazomethane quencher, Powers and Meister (37) successfully trapped carboxyphosphate as a stable trimethyl ester from the reaction of carbamyl phosphate synthetase. This experiment provided unambiguous proof of the phosphorylation of bicarbonate by ATP. It would seem that that the biotin-dependent carboxylases follow a similar mechanism, although to date, attempts to trap the intermediate in the same fashion from biotin carboxylase (38) and pyruvate carboxylase (39) have been unsuccessful.

If carboxyphosphate cannot be trapped from a biotin-dependent carboxylase, isosteric analogs of carboxyphosphate may provide further evidence of its intermediacy.

Phosphonoacetetic acid, a nonhydrolyzable analog, has been shown to inhibit pyruvate carboxylase (40), though the  $K_i$  does not indicate a high affinity (0.5 mM). Polakis *et al.* (41) discovered that biotin carboxylase catalyzes the phosphorylation of ADP by carbamyl phosphate as follows [Scheme 1.7]:



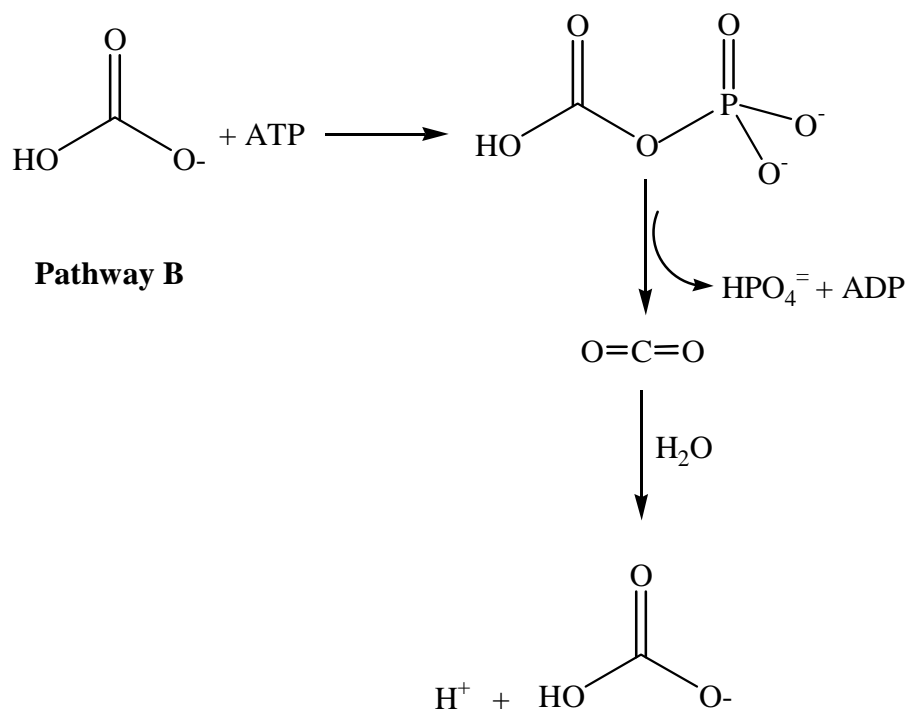
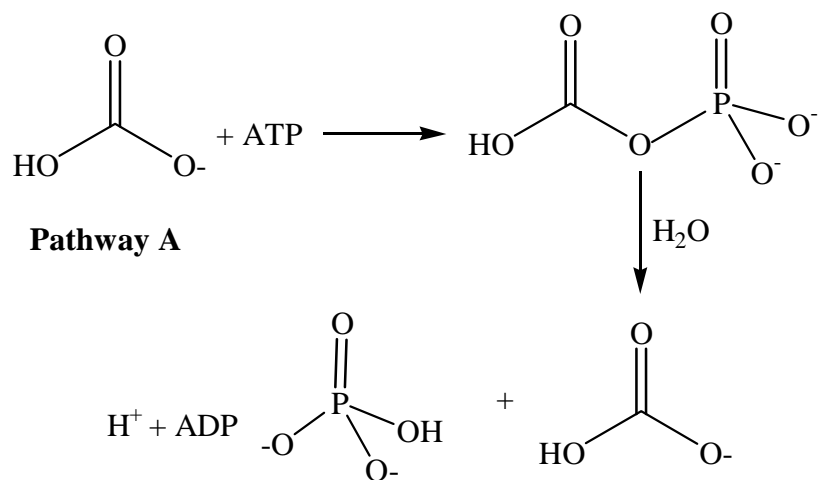
Scheme 1.7

This scheme represents the reverse of the physiological reaction, with carbamyl phosphate acting as an analog of carboxyphosphate. However, it can be easily argued that carbamyl phosphate acts as an analog of *O*-phosphobiotin, particularly in light of the fact that biotin is not required

for the ATP synthesis to proceed (42). Thus it seems that analogs provide tenuous support for the existence of carboxyphosphate.

Climont & Rubio (43) observed that *E. coli* biotin carboxylase has a slow bicarbonate-dependent ATPase activity in the absence of biotin, providing further evidence against the formation of the *O*-phosphobiotin intermediate. Subsequently, it was demonstrated that when ATP reacts with  $^{18}\text{O}$ -labeled bicarbonate, one of the labeled oxygens ends up in the product  $\text{P}_i$  (38). These two observations have provided by far the most definitive evidence against the requirement for biotin in the hydrolysis of ATP, which suggests that ATP reacts with bicarbonate first to form carboxyphosphate.

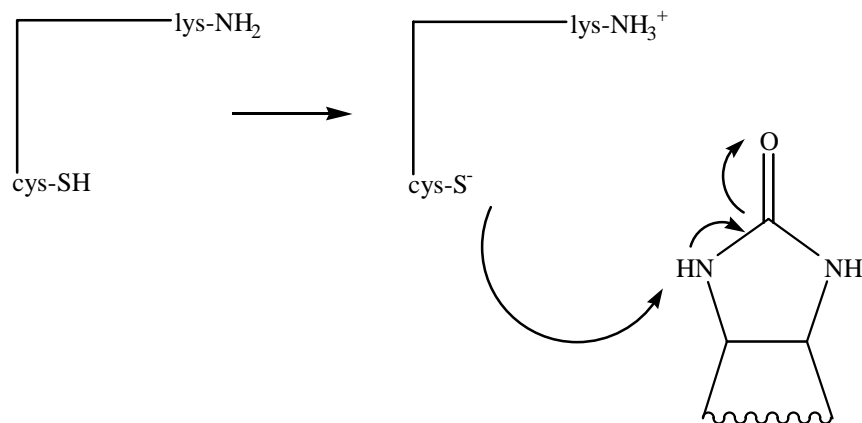
The next question, and one which has no unequivocal answer as of yet, is the identity of the carboxylating species. In other words, does biotin attack carboxyphosphate directly (Pathway A of scheme 1.8), or does carboxyphosphate collapse into  $\text{CO}_2$  and  $\text{P}_i$ , with subsequent attack on  $\text{CO}_2$  (Pathway B of scheme 1.8)? The advantage of the latter mechanism is that  $\text{CO}_2$  is a much better electrophile than carboxyphosphate and the enzyme would thus be able to utilize the abundant bicarbonate molecule to deliver a  $\text{CO}_2$  to biotin. This issue was addressed for carbamyl phosphate synthetase by Gibson *et al.* (44) in an elegant experiment designed to distinguish between an enzyme catalyzed dehydration of bicarbonate and a simple breakdown of carboxyphosphate into bicarbonate and  $\text{P}_i$ . In pathway A, the rate of formation of ADP and the proton should be equal because they are released simultaneously. This is due to the very short half-life of the highly activated carboxyphosphate, which is rapidly hydrolyzed. If the enzyme catalyzed the formation of  $\text{CO}_2$  from carboxyphosphate, as in pathway B, the rate of proton release would lag behind ADP formation, indicating a slow, non-enzyme catalyzed hydration of  $\text{CO}_2$ . The rates of ADP and proton release were in fact, equal, which strongly supports



Scheme 1.8

carboxyphosphate as the carboxylating entity for carbamyl phosphate synthetase (44). In light of the mechanistic homology of carbamyl phosphate synthetase with the biotin-dependent carboxylases, it does not seem unreasonable to suppose that this mechanism also occurs in any biotin-dependent carboxylase. Furthermore, several other enzymes of the ATP-grasp superfamily proceed not through a carboxyphosphate intermediate, but through an acylphosphate intermediate. Thus, direct attack on the acylphosphate would be the most plausible mechanism, suggesting that the reaction catalyzed by biotin carboxylase proceeds analogously.

Activation of Biotin. The low nucleophilicity of biotin could be greatly improved by deprotonation of the 1' nitrogen, resulting in an enolate which could then attack the carboxylating substrate. Possible mechanisms for 1' deprotonation have been investigated. The possibility that an active site cysteine and lysine could be an acid-base pair for removal of the proton looked particularly attractive due to the work of Werneburg and Ash, who demonstrated that *O*-phthalaldehyde inactivated pyruvate carboxylase (45). *O*-phthalaldehyde acts to cross-link lysine and cysteine residues, forming an inactive isoindole enzyme derivative. The inactivation was blocked by the substrates, suggesting that the putative essential Cys-Lys pair was located in the active site. The crystal structure of *E. coli* biotin carboxylase revealed that Cys 230 and Lys 238 in the active site pocket were separated by 4.2 Å, a distance which would permit the two residues to act as an ion pair and which would account for their cross-linkage by *O*-phthalaldehyde. Furthermore, Cys 230 and Lys 238 are strictly conserved among the biotin-dependent carboxylases, supporting the notion that they could play this essential role. Thus, a mechanism was proposed in which the ε-amino of Lys 238 deprotonates the sulfhydryl group of Cys 230. The thiolate anion can then abstract the proton from the 1' nitrogen of biotin while the protonated lysine stabilizes the negative charge on the enolate oxygen (Scheme 1.9).



Scheme 1.9

This mechanism was investigated by Levert *et al.* (42), who constructed the site-directed mutants C230A and K238Q of biotin carboxylase. While kinetic studies on K238Q revealed that Lys 238 plays an essential role in the carboxylation of biotin, the kinetics of the C230A mutant resembled wild type except for the elevated  $K_m$  ATP, which implicated Cys 230 in binding ATP. Moreover, the  $pK_a$  of Lys 238 was found to be 9.4 or higher, making it an unlikely candidate for a catalytic base. It was thus concluded that Cys 230 and Lys 238 do not act as an acid-base pair to activate biotin, and that Lys 238 instead orients the carboxyphosphate intermediate for efficient carboxyl transfer. To address the question of how the 1' nitrogen is deprotonated, Levert *et al.* suggested that one of the phosphate oxygens of carboxyphosphate could act in this capacity, an economical mechanism known as substrate-assisted catalysis (42).

### Objectives and Rationale for Study

While the chemical and mechanistic aspects of biotin carboxylase have been scrutinized thoroughly, precious little information is available on the roles of the active site residues. A more complete understanding of the catalytic mechanism would be gained through examination of the interactions between key amino acid residues and the substrates they contact. To this end,

site-directed mutagenesis was employed in two separate studies to explore the roles of the residues Arg 338, His 209, Glu 276, Met 169, Lys 159, and Lys 116.

To approach these studies, the first question that must be asked is, “What residues do you mutate?” and then “What do you mutate them to?” In the first study, the residues Lys 116, Lys 159, His 209 and Glu 276 were examined because of their high degree of conservation among the ATP-grasp enzymes. Moreover, structural data on several ATP-grasp enzymes suggested pertinent roles for the four residues based on their interactions with ATP. Thus, the residues were each mutated to an isosteric residue or alanine in order to minimize disruption of the protein structure. In the second study, Met 169 and Arg 338 were selected for mutagenesis due to their involvement in two human metabolic diseases caused by deficiencies in biotin-dependent carboxylases. In order to mimic the missense mutations that give rise to the disease states, the same (homologous) mutations were introduced into the *E. coli* biotin carboxylase model system. Thus, naturally occurring deleterious mutations provided the residue of interest as well as the residue change.

## References

1. Waheed A and Castric PA. (1977) *J Biol Chem* **252**, 1628-32.
2. Whitney PA and Cooper, TG. (1972) *J Biol Chem* **247**, 1349-53.
3. Hodson RC, Williams SK 2<sup>nd</sup>, and Davidson WR Jr. (1975) *J Bacteriol* **121**, 1022-35.
4. Guan X, Diez T, Prasad TK, Nikolau BJ, and Wurtele ES. (1999) *Arch Biochem Biophys* **362**, 12-21.
5. Jitrapakdee S and Wallace, JC. (2003) *Curr Protein Pept Sci* **4**, 217-229.
6. Wood HG and Kumar GK. (1985) *Ann N Y Acad Sci* **447**, 1-22.
7. Wright LD, Cresson EL, Skeggs, GR, Wood, TR, Peck RL, Wolf DE, and Folkers K. (1952) *J Am Chem Soc* **74**, 1996-2001.

8. Samols D, Thornton CG, Murtif VL, Kumar GK, Haase FC, and Wood HG. (1988) *J Biol Chem* **263**, 6461-4.
9. Wood HG, Shenoy BC, Kumar GK, Paranjape S, Murtif V, and Samols D. (1987) *Fed Proc* **46**, 2124 (abstr.)
10. Paranjape S, Shenoy BC, Kumar GK, Samols D, and Wood HG. (1988) *FASEB J* **2**, A998.
11. Wakil SJ, Titchener EB, and Gibson DM. (1958) *Biochim Biophys Acta* **29**, 225-6.
12. Norman E, DeSmet KA, Stoker NG, Ratledge C, Wheeler, PR, and Dale JW. (1994) *J Bacteriol* **176**, 2525-31.
13. Kimura Y, Miyake R, Tokumasu Y, and Sato M. (2000) *J Bacteriol* **182**, 5462-9.
14. Wakil SJ, Stoops JK, and Joshi, VC. (1983) *Ann Rev Biochem* **52**, 537-79.
15. Cronan JE Jr, and Waldrop GL. (2002) *Prog Lipid Res* **41** 407-435.
16. Davis MS and Cronan JE Jr. (2001) *J Bacteriol* **183**, 1499-503.
17. Li SJ and Cronan JE Jr. (1993) *J Bacteriol* **175**, 332-40.
18. James ES and Cronan JE Jr. (2003) *J Biol Chem*, in press.
19. Mitchelhill KI, Stapleton D, Gao G, House C, Michell B, Katsis F, Witters LA, and Kemp BE. (1994) *J Biol Chem* **269**, 2361-64.
20. Woods A, Munday MR, Scott J, Yang X, Carlson M, and Carling D. (1994) *J Biol Chem* **269**, 19509-15.
21. Moss J and Lane, DM. (1971) *Adv Enzymol Relat Areas Mol Biol* **35**, 321-442.
22. Witters LA, Widmer J, King AN, Fassihi K, and Kuhajda F. (1994) *Int J Biochem* **26**, 589-94.
23. Abu-Elheiga L, Brinkley WR, Zhong L, Chirala SS, Woldegiorgis G, and Wakil SJ. (2000) *Proc Natl Acad Sci USA* **97**, 1444-9.
24. Milgraum LZ, Witters LA, Pasternack GR, and Kuhajda, FP. (1997) *Clin Cancer Res* **3**, 2115-50.
25. Deng CX and Brodie SG (2000) *Bioessays* **22**, 728-37.
26. Magnard C, Bachelier R, Vincent A, Jaquinod M, Kieffer S, Lenoir GM and Venezia ND. (2002) *Oncogene* **21**, 6729-39.

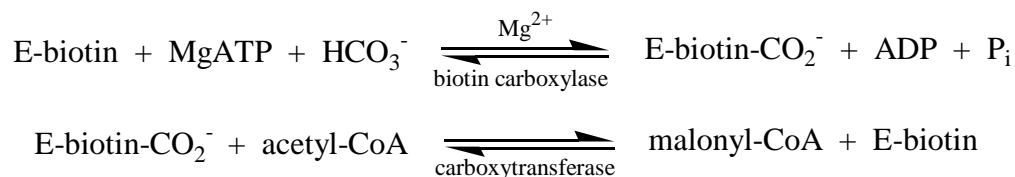
27. Abu-Elheiga L, Matzuk, MM, Abo-Hashema KAH, and Wakil SJ. (2001). *Science* **291**, 2613-16.
28. Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, and Kahn BB. (2002) *Nature* **415**, 339-43.
29. Waldrop GL, Rayment I, and Holden HM. (1994) *Biochemistry* **33**, 10249-56.
30. Thoden JB, Blanchard CZ, Holden HM, and Waldrop GL. (2000) *J Biol Chem* **275**, 16183-90.
31. Artymiuk PJ, Poirrette AR, Rice DW, and Willett P. (1996) *Nat Struct Biol* **3**, 128-32.
32. Galperin MY and Koonin EV (1997) *Protein Sci* **6**, 2639-43.
33. Thoden JB, Wesenberg G, Raushel FM, and Holden HM. (1999) *Biochemistry* **38**, 2347-57.
34. Northrop DB (1969) *J Biol Chem* **244**, 5808-19.
35. Calvin M and Pon NG. (1959) *J Cell Comp Physiol* **54**, 51-74.
36. Lynen F. (1967) *Biochem J* **102**, 381-400.
37. Powers SG and Meister A. (1976) *Proc Natl Acad Sci USA* **73**, 3020-24.
38. Ogita T and Knowles JR. (1988) *Biochemistry* **27**, 8028-33.
39. Wallace JC, Phillips NB, Snowswell MA, Goodall JG, Attwood PV and Keech DB. (1985) *Ann N Y Acad Sci* **447**, 169-88.
40. Ashman LK and Keech DB. (1975) *J Biol Chem* **250**, 14-21.
41. Polakis SE, Guchhait RB and Lane MD. (1972) *J Biol Chem* **247**, 1335-7.
42. Levert KL, Lloyd RB, and Waldrop GL. (2000) *Biochemistry* **39**, 4122-28.
43. Climent I and Rubio V. (1986) *Arch Biochem Biophys* **251**, 465-70.
44. Gibson GE, Mullins LS, and Raushel FM. (1998) *Bioorg Chem* **26**, 255-68.
45. Werneburg BG and Ash DE. (1993) *Arch Biochem Biophys* **303**, 214-21.

## CHAPTER 2

### SITE-DIRECTED MUTAGENESIS OF ATP BINDING RESIDUES OF *E. COLI* BIOTIN CARBOXYLASE: INSIGHT INTO THE MECHANISM OF CATALYSIS\*

#### Introduction

Acetyl-CoA carboxylase catalyzes the first committed step in long-chain fatty acid synthesis, namely the formation of malonyl-CoA from acetyl-CoA, MgATP, and bicarbonate. Found in all plants, animals, and bacteria, this enzyme is biotin dependent, with the following 2-step reaction mechanism (Scheme 2.1):



Scheme 2.1

The *Escherichia coli* form of this enzyme consists of three separable components. The biotin carboxylase component catalyzes the first half reaction which involves the phosphorylation of bicarbonate to form a carboxyphosphate intermediate, followed by the transfer of the carboxyl group to the 1' nitrogen of biotin (2). The carboxyltransferase component catalyzes the second half reaction. *In vivo*, the biotin molecule is linked to the biotin carboxyl carrier protein through an amide bond to a specific lysine residue. Both biotin carboxylase and carboxyltransferase retain activity in the absence of the other two components and will also use free biotin as a substrate (3). The crystal structure of the biotin carboxylase component has been solved and is the only three-dimensional structure of a biotin-dependent carboxylase, making it the paradigm for structure-function analysis of this class of enzymes (4).

---

Reprinted with permission from *The Journal of Biological Chemistry*. Copyright 2001, American Society of Biochemistry and Molecular Biology.

Two years after the solution of the crystal structure, Artymiuk *et al.* observed that biotin carboxylase had a strong structural homology to glutathione synthetase and D-ala D-ala ligase (5). Despite the remarkable similarity in the three-dimensional structures of biotin carboxylase, D-ala D-ala ligase and glutathione synthetase, there is only an 11% primary sequence identity between the three enzymes (5). Although biotin carboxylase is metabolically unrelated to these two enzymes, all three enzymes are mechanistically homologous in that they catalyze the ATP-dependent ligation of a carboxylate-containing substrate to an amine-containing substrate via formation of an acylphosphate intermediate (5, 6). Structural similarity between the three enzymes includes a common three-domain architecture in which the flexible central domain extends away from the main body of the protein. The crystal structure of biotin carboxylase was originally determined in the absence of any ligands or substrate analogs (4), and its central domain (known as the B-domain) was in the “open” conformation, extending far out from the main body of the enzyme. In contrast, the structures of D-ala D-ala ligase and glutathione synthetase were solved in the presence of ADP and ATP, respectively, which revealed that the central domain forms a “lid” that clamps down over the active site upon nucleotide binding (7, 8). Using the structures of D-ala D-ala ligase and glutathione synthetase, Artymiuk *et al.* identified several active site residues of biotin carboxylase as potentially important for catalysis; among these were Lys 116, Lys 159, His 209, Lys 238, Glu 276, Glu 288 and Asn 290. Soon after the observations of Artymiuk *et al.*, the three dimensional structure of carbamyl phosphate synthetase was reported and found to be homologous to biotin carboxylase, D-ala D-ala ligase, and glutathione synthetase (9). The structural and mechanistic similarity of all four enzymes suggested they were linked through evolution and thus they became the charter members of the ATP-grasp family of enzymes. The name “ATP-grasp” derives from the novel nucleotide-

binding fold observed in these enzymes. The ATP-grasp family of enzymes expanded even further to include several enzymes involved in purine biosynthesis based on a position specific iterative BLAST sequence alignment (6, 10). The three-dimensional structure of two of these enzymes, *N*<sup>5</sup>-carboxyaminoimidazole ribonucleotide synthetase (11) and glycinamide ribonucleotide transformylase, (12) have been determined with nucleotides bound.

The sequence analysis studies identified several residues as being strictly conserved throughout the entire ATP-grasp family of enzymes. Not surprisingly, the conserved residues in biotin carboxylase were Lys 116, Lys 159, His 209, Glu 276, Glu 288, and Asn 290. Site-directed mutagenesis studies of Glu 288 and Asn 290 confirmed that these two residues were indeed important for catalysis (13). In fact, mutation of Glu 288 to lysine resulted in a completely inactive mutant (14). Recently, Thoden *et al.* determined the crystal structure of the inactive mutant of biotin carboxylase, E288K, cocrystallized with ATP. The structure showed that the B-domain of biotin carboxylase does exhibit the characteristic “trap door” closure in the presence of nucleotide, with some atoms moving by more than 8 Å. (14). As expected, comparison of the structure of the mutant biotin carboxylase-ATP complex with the structures of the other enzymes of the ATP-grasp superfamily revealed a significant degree of homology. For example, Lys 116 of biotin carboxylase and the residue homologous to Lys 116 in the other ATP-grasp enzymes was found to interact with ATP. However, there were also some notable differences between the structure of biotin carboxylase and the structures of the other ATP-grasp enzymes. Namely, the biotin carboxylase crystal structure suggested that Lys 159, His 209 and Glu 276 did not interact with ATP while the structures of the other ATP-grasp enzymes indicated these residues did interact with ATP. Thus, the objective of this study is to test the hypothesis

that residues Lys 116, Lys 159, His 209 and Glu 276 of biotin carboxylase are involved in binding ATP.

## **Materials and Methods**

Chemicals and Enzymes. Sodium bicarbonate labeled with  $^{14}\text{C}$  was from Amersham and had a specific activity of 0.1 mCi/mmol. His-binding resin was from Novagen. Pyruvate kinase was from Roche Molecular Biochemicals. Restriction grade bovine thrombin was from Enzyme Research Laboratories. Primers were synthesized by Life Technologies, Inc. All other reagents were from Sigma or Aldrich. The growth and purification of wild type and mutant forms of biotin carboxylase were performed as previously described (13).

Site-directed Mutagenesis. Site-directed mutagenesis of biotin carboxylase was carried out by the PCR method of overlap extension as previously described (13). The following mutants were constructed: H209A, E276Q, K159Q, K116Q, and K116A. The pairs of internal mutagenic primers used to make each site-directed mutant can be found in Table 2.1. The entire gene of each mutant was sequenced to confirm that the desired mutation was made and that no other mutations were incorporated during polymerase chain reaction.

Enzymatic Assays. The rate of ATP hydrolysis by biotin carboxylase in the absence or presence of biotin was measured spectrophotometrically by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase and monitoring the oxidation of NADH at 340 nm. Each assay contained 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 units of pyruvate kinase, 18 units of lactate dehydrogenase, and 100 mM HEPES at pH 8. To ensure the formation of the MgATP complex,  $\text{MgCl}_2$  was included at concentrations at least twice that of the highest concentration of ATP in each assay. Since the  $K_m$  for biotin is high (134 mM), the ionic strength

**Table 2.1. Primers Used for Site-Directed Mutagenesis of Biotin Carboxylase**

<b>Enzyme</b>	<b>DNA Sequence</b>
K116Q	5' GATTGCGGATACTT <u>G</u> GTCGCCCATCAG 3' 5' CTGATGGGCGAC <u>C</u> AAGTATCCGCAATC 3'
K116A	5' GATTGCGGATACT <u>G</u> CGTCGCCCATCAG 3' 5' CTGATGGGCGAC <u>C</u> AGTATCCGCAATC 3'
K159Q	5' CCGCCGGAGGCTT <u>G</u> GATAATCACCG 3' 5' CGGTGATTATC <u>C</u> AAGCCTCCGGCGG 3'
H209A	5' CTGAATCTCGAC <u>G</u> GCGGAGGATTTTC 3' 5' GAAAATCCTCGCG <u>C</u> CGTCGAGATTCAG 3'
E276Q	5' TCGAACAGGAACT <u>G</u> GAAAGTACCTG 3' 5' CAGGTACTTT <u>C</u> AGTTCCTGTTCGA 3'

The underlined bases indicate the nucleotide positions that were changed.

of the reaction mixture was held constant with KCl when the initial velocities were measured as a function of biotin concentration.

For experiments in which bicarbonate was varied, all solutions (except for coupling enzymes, which were diluted into degassed buffers) were degassed in order to lower the level of endogenous bicarbonate (13) and stored in septum vials capped with rubber septa. All assay reactions were performed in a total volume of 1 mL and included the following components: 60 mM biotin, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 21 units of pyruvate kinase, 35 units of lactate dehydrogenase, and 100 mM HEPES at pH 8.

For experiments in which the concentration of magnesium was varied, the N-terminal His-tag on biotin carboxylase was removed by thrombin cleavage to eliminate the possibility of magnesium binding to the His-tag (13). A ratio of 2 units of thrombin per unit of biotin carboxylase was used.

The rate of ATP synthesis from MgADP and carbamyl phosphate was determined spectrophotometrically. The formation of ATP was coupled to hexokinase and glucose-6-phosphate dehydrogenase, with the production of NADPH monitored at 340 nm. Each assay contained 0.5 mM glucose, 0.4 mM NADP, 2.5 units of hexokinase, 2.5 units of glucose-6-phosphate dehydrogenase, 100 mM KCl, and 100 mM HEPES at pH 8. To ensure the formation of the MgADP complex, MgCl<sub>2</sub> was included and held at concentrations at least twice that of the highest concentration of ADP in the assay.

Initial velocities were measured using a Uvikon 810 (Kontron Instruments) spectrophotometer interfaced to a PC equipped with a data acquisition program. The temperature of the reactions was maintained at 25<sup>0</sup> C by a circulating water bath. All reactions were initiated by the addition of enzyme. Kinetic parameters were calculated per active site

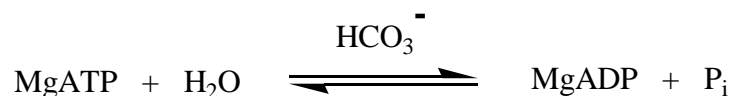
using a MW of 50,000 daltons for the biotin carboxylase monomer (biotin carboxylase exists as a homodimer).

To determine if there was a stoichiometric production of ADP and carboxybiotin, the amount of carboxybiotin produced by biotin carboxylase was determined using a  $^{14}\text{C}$  fixation assay and compared to the production of ADP as previously described (13). The reaction mixtures contained 20 mM ATP, 70 mM bicarbonate, 100 mM biotin, 50 mM  $\text{MgCl}_2$ , and 100 mM HEPES at pH 8 in a total volume of 0.5 mL.

Data Analysis. The  $K_m$  and  $V_{max}$  parameters were determined by nonlinear regression analysis of the velocity versus [substrate] data to the Michaelis-Menten equation using the program Enzfitter.

## Results

Bicarbonate-Dependent ATPase Reaction. In the absence of biotin, biotin carboxylase from *E. coli* catalyzes a bicarbonate-dependent ATP hydrolysis (Scheme 2.2):



Scheme 2.2

This reaction has been proposed to occur via formation of carboxyphosphate, which rapidly decomposes in the absence of biotin (15). The Michaelis constants for ATP and the maximal velocity of this partial reaction were determined for the wild-type enzyme and four mutant enzymes of biotin carboxylase (Table 2.2). All four mutants showed no significant change in  $V_{max}$  when compared to the wild-type enzyme. However, the  $K_m$  for ATP for all four mutants increased between 40- and 90- fold.

The  $K_m$  for bicarbonate for each of the mutants was determined at fixed, nonsaturating levels of ATP and biotin. Since it was not possible to achieve saturation with biotin or with

**Table 2.2. Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction**

	$K_m$ $\text{HCO}_3^-$ (mM)	$K_m$ ATP (mM)	$V_{\max}$ ( $\text{min}^{-1}$ )	$V/K_{\text{ATP}}$ ( $\text{mM}^{-1} \text{min}^{-1}$ )
<b>Wild Type</b>	$1.1 \pm 0.3$	$0.08 \pm 0.01^a$	$0.073 \pm 0.001^a$	$0.9 \pm 0.1^a$
<b>H209A</b>	$16.7 \pm 1.0$	$5.7 \pm 1.2$	$0.043 \pm 0.004$	$0.008 \pm 0.002$
<b>E276Q</b>	$1.9 \pm 0.1$	$3.0 \pm 0.2$	$0.072 \pm 0.002$	$0.024 \pm 0.002$
<b>K159Q</b>	$1.8 \pm 0.2$	$7.4 \pm 1.4$	$0.087 \pm 0.009$	$0.012 \pm 0.004$
<b>K116Q</b>	$1.3 \pm 0.1$	$4.2 \pm 0.8$	$0.075 \pm 0.007$	$0.018 \pm 0.005$

The kinetic parameters were determined by varying [ATP] at a constant saturating level of bicarbonate or by varying  $[\text{KHCO}_3]$  while holding ATP at 25 mM. For all mutants except H209A, 60 mM biotin was included in assays in which bicarbonate was varied. The residual bicarbonate in the solutions prevented kinetic analysis unless biotin was added to the reaction to stimulate rates of catalysis. Biotin was excluded from the assays for H209A because the  $K_m$  for bicarbonate was high enough that accurate rates of catalysis could be obtained at subsaturating concentrations of bicarbonate. Standard errors on  $K_m$  and  $V_{\max}$  were calculated from nonlinear regression analysis. The error on  $V/K$  was calculated by standard propagation of the errors from  $K_m$  and  $V_{\max}$ . <sup>a</sup> Data taken from ref. 13.

ATP, the  $K_m$  values for bicarbonate are apparent  $K_m$  values. The apparent  $K_m$  values for wild type biotin carboxylase and the four mutants are shown in Table 2.2. The mutations did not affect significantly the apparent  $K_m$  for any mutant except that of H209A, where the  $K_m$  was 15 times greater than that of wild type.

Biotin-Dependent ATPase Reaction. In the presence of biotin, biotin carboxylase from *E. coli* catalyzes the phosphorylation of bicarbonate by ATP to form a carboxyphosphate intermediate. The carboxyl group is then transferred from carboxyphosphate to biotin to form carboxybiotin. When ATP hydrolysis activity was examined in the presence of biotin, the  $K_m$  values for biotin for all mutants were not significantly different compared to the wild type enzyme (Table 2.3). The  $K_m$  values for biotin are apparent because it was not possible to saturate with ATP. The largest change in  $K_m$  was exhibited by H209A, but was less than 10 fold. However,  $V_{max}$  for three of the four mutants decreased significantly between 30- and 200-fold. The one exception is K116Q, for which a  $K_m$  value could not be obtained; the activity of this mutant did not increase with the addition of biotin over a range of 1-300 mM. To further investigate the role of this lysine residue in the biotin-dependent ATPase reaction, a K116A mutant was constructed and the initial velocity as a function of biotin concentration was measured. The  $K_m$  for biotin for the K116A mutant was  $147 \pm 12$  mM while the maximal velocity was  $1.03 \pm 0.04$  min<sup>-1</sup>. The effect of this mutation was similar to that of the other three in that the  $V_{max}$  rather than the  $K_m$  was significantly altered with respect to wild type. These data suggest that the lack of stimulation of ATP hydrolysis by biotin for K116Q reflects a function of the mutant glutamine residue rather than of the role of the native lysine residue.

Since the ATPase assay measures the production of ADP in the presence and absence of biotin, the question still remained as to whether carboxybiotin was being produced by the mutant

**Table 2.3. Kinetic Parameters for the Biotin-Dependent ATPase Reaction**

	<b>K<sub>m</sub> biotin (mM)</b>	<b>V<sub>max</sub> (min<sup>-1</sup>)</b>	<b>V/K<sub>biotin</sub> (mM<sup>-1</sup> min<sup>-1</sup>)</b>
<b>Wild Type<sup>a</sup></b>	134 ± 12	78.6 ± 7	0.59 ± 0.11
<b>H209A</b>	1234 ± 117	2.49 ± 0.17	0.0020 ± 0.0003
<b>E276Q</b>	137 ± 24	0.90 ± 0.06	0.006 ± 0.001
<b>K159Q</b>	125 ± 10	0.37 ± 0.01	0.0030 ± 0.0004
<b>K116Q</b>	*	*	*

The kinetic parameters were determined by varying [biotin] at constant saturating levels of bicarbonate and 25 mM ATP. The standard errors on V<sub>max</sub> and K<sub>m</sub> were calculated from nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from K<sub>m</sub> and V<sub>max</sub>. Because the ATPase activity for K116Q responded very little to concentrations of biotin between 20 and 300 mM, kinetic parameters could not be obtained for K116Q. <sup>a</sup> Data taken from ref. 13.

enzymes. In other words, is there a 1:1 stoichiometry for the formation of ADP and carboxybiotin or is the hydrolysis of ATP uncoupled from the formation of carboxybiotin? If the 1:1 ratio were altered, this would suggest that the mutations had affected the carboxyl transfer step. The ratio of carboxybiotin to ADP produced during the ATPase reaction for wild type and the four mutants was determined (Table 2.4). All four mutants produced a ratio of carboxybiotin to ADP that was nearly 1:1. These results indicate that the mutations did not prevent the production of carboxybiotin and therefore the carboxyl transfer step had not been uncoupled from the hydrolysis of ATP.

ATP Synthesis Reaction. Biotin carboxylase from *E. coli* has been shown to catalyze the transfer of the phosphoryl group of carbamyl phosphate to ADP to form ATP and carbamate as follows.



Scheme 2.3

The carbamate rapidly decomposes to carbon dioxide and ammonia. Scheme 2.3 represents the reverse of Scheme 2.2 with carbamyl phosphate acting as an analog of the putative carboxyphosphate intermediate. Although biotin does not participate in the chemistry of this reaction, its presence does stimulate the rate of phosphoryl transfer (16).

The kinetic parameters for the ATP synthesis reaction were determined in the absence of biotin (Table 2.5). The mutations did not have a significant effect on the  $K_m$  for either carbamyl phosphate or ADP. However, a modest decrease in  $V_{max}$  of 4-fold or less was observed.

To test the ability of biotin to stimulate the phosphoryl transfer reaction of the wild type and four mutants of biotin carboxylase, initial velocities were measured at a saturating

**Table 2.4. Ratio of ADP to Carboxybiotin Production**

<b>Wild Type<sup>a</sup></b>	$0.96 \pm 0.18$
<b>H209A</b>	$1.2 \pm 0.1$
<b>E276Q</b>	$0.86 \pm 0.01$
<b>K159Q</b>	$0.78 \pm 0.16$
<b>K116Q</b>	$1.1 \pm 0.1$

The amounts of ADP and carboxybiotin formed over a 1 hour period were quantified as described in Materials and Methods. <sup>a</sup> Data taken from ref. 13.

**Table 2.5. Kinetic Parameters for the ATP Synthesis Reaction**

	$K_m$ CbmP (mM)	$K_m$ ADP (mM)	$V_{max}$ ( $\text{min}^{-1}$ )	$V/K_{ADP}$ ( $\text{mM}^{-1} \text{min}^{-1}$ )
<b>Wild Type<sup>a</sup></b>	$4.8 \pm 0.2$	$0.19 \pm 0.01$	$0.31 \pm 0.01$	$1.6 \pm 0.1$
<b>H209A</b>	$7.3 \pm 0.5$	$0.39 \pm 0.07$	$0.12 \pm 0.01$	$0.31 \pm 0.08$
<b>E276Q</b>	$2.3 \pm 0.1$	$0.42 \pm 0.01$	$0.087 \pm 0.001$	$0.21 \pm 0.01$
<b>K159Q</b>	$3.0 \pm 0.6$	$0.93 \pm 0.08$	$0.12 \pm 0.01$	$0.13 \pm 0.02$
<b>K116Q</b>	$5.0 \pm 0.7$	$0.31 \pm 0.05$	$0.077 \pm 0.003$	$0.25 \pm 0.05$

The kinetic parameters were determined by varying carbamyl phosphate concentration (CbmP) at constant saturating levels of ADP or varying [ADP] at constant saturating levels of CbmP. The standard errors on  $K_m$  and  $V_{max}$  were calculated from nonlinear regression analysis. The error on  $V/K$  was calculated by standard propagation of the errors on  $K_m$  and  $V_{max}$ . <sup>a</sup> Data taken from ref. 24.

concentrations of ADP and carbamyl phosphate, both in the absence and presence of 60 mM biotin (Table 2.6). The degree of stimulation of the ATP synthesis activity by biotin was decreased 10 fold by the E276Q mutation, while the H209A mutant showed no significant decrease in stimulation. Both K116Q and K159Q showed a decrease of ~2.5-fold compared to wild type.

Magnesium Assay. Biotin carboxylase requires two equivalents of magnesium for activity. One equivalent is complexed to ATP while the role of the other equivalent is unknown. The effect of the four mutations on the ability of magnesium to stimulate the biotin-dependent ATPase activity of wild type and mutant biotin carboxylase was evaluated by measuring initial velocity as a function of [MgCl<sub>2</sub>]. All four mutants exhibited a dependence on MgCl<sub>2</sub> similar to that of wild type. This suggests that these mutations did not affect the enzyme's affinity for magnesium (Figure 2.1).

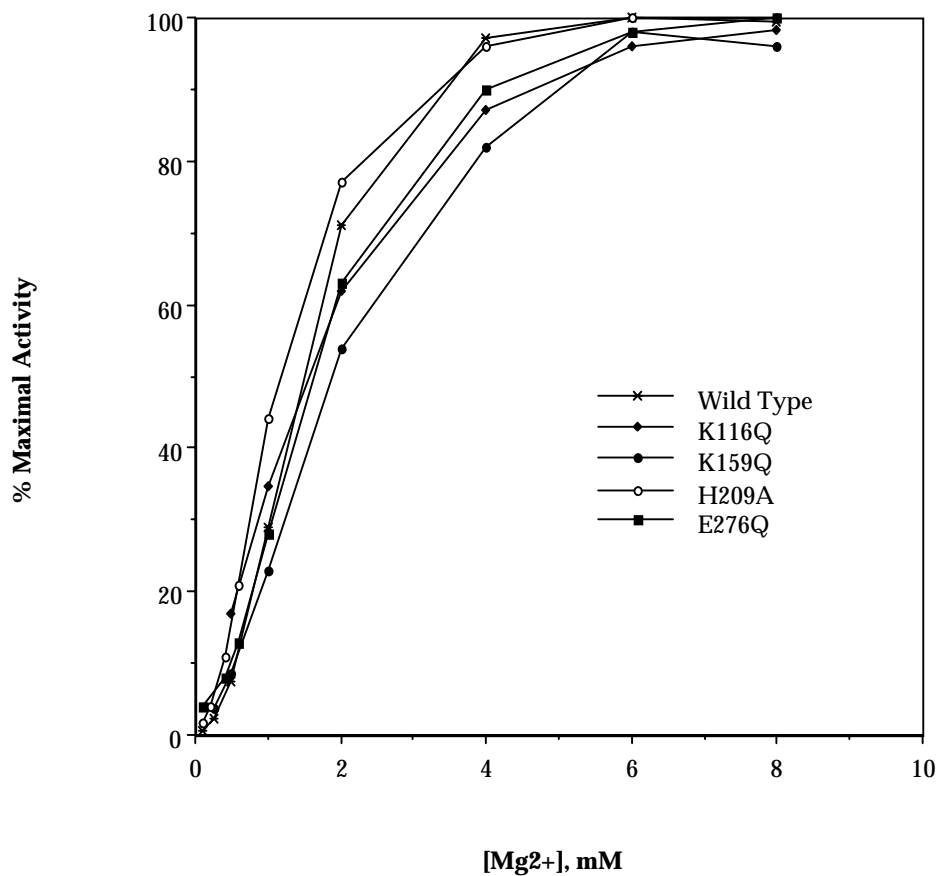
## **Discussion**

The initial objective of this study was to test the hypothesis that four residues of biotin carboxylase, Lys 116, Lys 159, Glu 276, and His 209, were involved in binding ATP. Each of the corresponding site-directed mutants displayed an elevated  $K_m$  value for ATP relative to the wild type value. This suggests that all four conserved active site residues bind ATP. For the K116Q mutant, the increased  $K_m$  value for ATP is consistent with the three-dimensional structure of biotin carboxylase with ATP bound as well as with the three-dimensional structures of other ATP-grasp enzymes bound to ADP or AMPPNP. The crystal structure of biotin carboxylase complexed with ATP revealed an electrostatic interaction between the  $\epsilon$ -amino group of Lys 116 and the  $\alpha$ -phosphoryl oxygen of ATP (14). As shown in Table 2.7, the residues homologous to Lys 116 in enzymes of the ATP-grasp family also interacted with the

**Table 2.6. Stimulation of ATP Synthesis Reaction**

	<b>Stimulation factor</b>
<b>Wild Type</b>	25
<b>H209A</b>	21
<b>E276Q</b>	2
<b>K159Q</b>	9
<b>K116Q</b>	10

The initial velocity of the ATP synthesis reaction was measured both in the presence and absence of 60 mM biotin. All reactions contained saturating levels of ADP, carbamyl phosphate, and magnesium. The stimulation factor is the ratio of the rate with biotin to the rate without biotin.



**Figure 2.1.** The effect of magnesium on the ATPase activity of wild type biotin carboxylase and the four mutants: K116Q, K159Q, H209A and E276Q. Initial velocities were measured at constant levels of ATP, bicarbonate, and biotin with increasing amounts of MgCl<sub>2</sub>. The degree of stimulation of the reaction by MgCl<sub>2</sub> is expressed as a percentage of the maximum rate measured for each of the mutants and wild type.

oxygens of the  $\alpha$  or  $\beta$  phosphates, as determined by crystallography. Moreover, mutation of the homologous residue in the carboxyphosphate domain of carbamyl phosphate synthetase resulted in a 5-fold increase in the  $K_m$  for ATP (17).

Although all the available crystallographic and kinetic data implicate Lys 116 in binding an  $\alpha$  or  $\beta$  phosphate oxygen, roles for the other three residues are not as well defined. Site-directed mutagenesis data for biotin carboxylase and the crystal structures of other ATP-grasp enzymes suggest differing roles for Lys 159, His 209, and Glu 276. First, the  $\epsilon$ -amino group of Lys 159 was not implicated in ATP binding based on the crystal structure of biotin carboxylase complexed with ATP. However, the residues homologous to Lys 159 in other ATP-grasp enzymes have been shown to interact with the nucleotide (Table 2.7), which supports the 90-fold increase in  $K_m$  for ATP in the K159Q mutant of biotin carboxylase. Site-directed mutagenesis of the homologous residues of carbamyl phosphate synthetase (carboxyphosphate domain) and D-alanine D-alanine ligase revealed a  $K_m$  for ATP that was 31-fold and 50-fold higher than the wild type value, respectively (17, 18). Together these data implicate Lys 159 in binding to ATP.

Second, the crystal structure of biotin carboxylase complexed with ATP showed that the imidazole group of His 209 is about 4Å from the hydroxyl groups of the ribose of ATP, whereas the crystal structures of other ATP-grasp enzymes showed that the residue homologous to His 209 hydrogen bonded to the 2' and 3' hydroxyl groups of the ribose (Table 2.7). As with K159Q, the  $K_m$  for ATP in the H209A mutant of biotin carboxylase was elevated 70-fold relative to wild type. Again, the structural data from other ATP-grasp enzymes and the site-directed mutagenesis results of biotin carboxylase strongly suggest that His 209 interacts with ATP, presumably with the hydroxyl groups of ribose.

**Table 2.7. Homologous residues in ATP-grasp enzymes**

<b>Residue in Biotin Carboxylase</b>	<b>D-ala D-ala Ligase<sup>b</sup></b>	<b>Carbamyl Phosphate Synthetase, carboxyphosphate domain<sup>c</sup></b>	<b>Carbamyl Phosphate Synthetase, carbamyl phosphate domain<sup>c</sup></b>	<b>N<sup>5</sup>-Carboxyaminoimidazole Ribonucleotide Synthetase<sup>d</sup></b>	<b>Glycinamide Ribonucleotide Transformylase<sup>e</sup></b>
<b>K116</b>	<b>K97</b> hydrogen bonds with $\alpha$ and $\beta$ phosphoryl groups of ATP	<b>R129</b> hydrogen bonds with $\alpha$ and $\beta$ phosphoryl groups of ATP	<b>R675</b> hydrogen bonds with $\beta$ phosphoryl group of ATP	<b>R80</b> hydrogen bonds with $\alpha$ and $\beta$ phosphoryl group of ATP	<b>R114</b> hydrogen bonds with $\beta$ phosphoryl group of ATP
<b>K159</b>	<b>K144</b> hydrogen bonds with $\alpha$ phosphate group	<b>R169</b> hydrogen bonds with $\alpha$ phosphate group	<b>R715</b> hydrogen bonds with $\alpha$ phosphate group	<b>K120</b> interacts with $\alpha$ phosphoryl and N7 of adenine	<b>K155</b> hydrogen bonds with $\alpha$ phosphate group
<b>H209</b>	<b>E187</b> hydrogen bonds to ribose hydroxyl groups	<b>E215</b> hydrogen bonds to ribose hydroxyl groups	<b>E761</b> hydrogen bonds to ribose hydroxyl groups	<b>E161</b> hydrogen bonds to ribose hydroxyl groups	<b>E203</b> hydrogen bonds to ribose hydroxyl groups
<b>E276</b>	<b>D257</b> coordinated to magnesium	<b>Q285</b> hydrogen bonds with $\alpha$ phosphate group	<b>Q829</b> coordinated to manganese	<b>E226</b> coordinated to magnesium	<b>E267</b> coordinated to magnesium

These enzymes were chosen for structural comparison with biotin carboxylase because of their known three-dimensional structures with bound nucleotide or nucleotide analog. <sup>a-d</sup> Data taken from Refs. 6, 27, 11, and 12, respectively.

Third, when the three-dimensional structure of biotin carboxylase with ATP was modeled with bound biotin, the ureido ring of biotin was located near the side chain of Glu 276 (14). However, three-dimensional structures of other ATP-grasp enzymes showed that the residue homologous to Glu 276 coordinated to either magnesium or manganese, and that the divalent cation in each structure coordinated to the phosphoryl oxygens of the  $\gamma$  phosphate group (Table 2.7). Site-directed mutagenesis of Glu 276 in biotin carboxylase indicated no change in the  $K_m$  for biotin but a nearly 40-fold increase in the  $K_m$  for ATP. This result supports the hypothesis that Glu 276 coordinates to a magnesium ion in biotin carboxylase and that mutation of Glu 276 distorts the binding of magnesium and in turn the binding of ATP. It should be noted that while the mutation of Glu 276 altered the binding of magnesium ion to the residue, it does not appear to change the affinity of magnesium for the enzyme (Figure 2.1). Additionally, when the homologous residue in the carboxyphosphate domain of carbamyl phosphate synthetase was mutated to alanine, the  $K_m$  for ATP for the resulting mutant was 19-fold higher than wild type carbamyl phosphate synthetase (17). Again, the mutagenesis data on biotin carboxylase were consistent with the structures and mutagenesis data of the other ATP-grasp enzymes, and inconsistent with the biotin carboxylase structure complexed with ATP.

The discrepancies described above can be explained by noting that the biotin carboxylase used in the crystal structure complexed with ATP contained a mutation of a critical residue (E288K) leading to a complete loss of activity. Although this mutation crippled the ability of the enzyme to hydrolyze ATP, it allowed crystals to be grown in the presence of ATP. The structural data from the other ATP-grasp enzymes in Table 2.7 were obtained from wild type protein. Furthermore, the crystal structure of biotin carboxylase does not contain a divalent cation coordinated to ATP. Thus, the crystal structure of the E288K mutant form of biotin

carboxylase complexed with ATP may not be a completely accurate description of the binding of ATP to this enzyme, which would explain the disagreement between the mutagenesis data and the crystal structure. Moreover, the evidence that ATP is bound incorrectly in the E288K mutant of biotin carboxylase may explain, at least in part, the lack of activity for the E288K mutant and provide insight into why the maximal velocities of the biotin-dependent ATPase activity for the four mutants characterized in this paper are significantly decreased.

While the increase in the Michaelis constants of the four mutants of biotin carboxylase strongly suggests that these residues are involved in binding ATP, the concomitant decrease in their maximal velocities indicates that these residues also play a role in catalysis. If these four residues were solely involved in binding ATP, then the corresponding mutants should have the same maximal velocity as wild type biotin carboxylase. This brings us to the question of what role these residues could play in catalysis that would be consistent with their role in binding ATP. Any discussion of the catalytic roles of these residues must begin with the observation that biotin carboxylase exhibits the phenomenon of substrate-induced synergism with respect to biotin (13). That is, in the absence of biotin, the enzyme will cleave ATP into ADP and  $P_i$  in a bicarbonate-dependent manner, albeit at a very slow rate. However, in the presence of biotin, the rate of ATP hydrolysis increases 1100-fold. Thus, the hydrolysis of ATP is synergistic with the binding of biotin. A possible explanation for substrate-induced synergism in biotin carboxylase that is consistent with the data presented in this paper is that in the absence of biotin, ATP binds to the enzyme in a large number of non-productive modes, which is manifested as a low maximal velocity. However, upon the binding of biotin to biotin carboxylase, the number of non-productive binding modes of ATP is reduced, allowing for a more reactive alignment of the substrates. This phenomenon is manifested as a dramatic increase in the maximal velocity.

Recently, a more sophisticated version of this concept has been proposed (19, 20). In the current theory, the enzyme molecule pre-organizes the active site to allow the substrates to form “near attack conformers” or NAC for short. A NAC refers to the juxtaposition of the substrates in the ground state such that they closely resemble the transition state. The effect of mutating any of the four residues in this study would be a shift in the active site geometry which would possibly reduce the number of near attack conformers. This is manifested as a significantly reduced maximal velocity for the mutant enzyme. Biotin cannot properly cause the alignment of ATP for the reaction because of greater conformational flexibility of ATP in the active site due to the mutation. This concept of greater conformational flexibility of ATP is supported by both the increase in  $K_m$  for ATP in each of the mutant enzymes and the presumably incorrect binding of ATP in the active site of the inactive E288K mutant. Recall that none of the mutants affected the carboxyl transfer from carboxyphosphate to biotin, yet the  $V_{max}$  values were significantly decreased. Thus, the fact that these four ATP-binding residues are conserved throughout the ATP-grasp superfamily of enzymes further attests to the notion that binding interactions and correct positioning of the substrates appear to play the dominant role in catalysis by biotin carboxylase.

The question is now how does biotin organize the substrates into a more reactive conformation. A conformational change (*i.e.*, induced fit) in biotin carboxylase upon biotin binding could explain the large increase in rate for ATP hydrolysis. However, the major conformational change in biotin carboxylase occurs upon the binding of ATP (14), which binds to the enzyme first before bicarbonate and biotin (21). A crystal structure of biotin carboxylase with only biotin bound showed no difference in conformation compared with the unliganded structure of the enzyme (4). The lack of a large conformational change in biotin carboxylase

upon binding biotin is consistent with the high  $K_m$  for biotin (134 mM); using this value as an apparent binding constant for biotin, a relatively low binding energy of 1.2 kcal/mole is obtained. The low binding energy of biotin to the enzyme is not suggestive of a large conformational change.

How then is biotin able to stimulate the rate of ATP hydrolysis, if not via a conformational change? Perhaps biotin only promotes very small changes in the enzyme that result in the alignment of substrates for catalysis. To this end, recent studies on hydrogen tunneling in dehydrogenases have found a correlation between protein dynamics and enzymatic activity (22, 23). Particularly intriguing is the case of isocitrate dehydrogenase, to which the binding of its substrate, isocitrate, induces shifts of less than an angstrom in the amino acid side chains of the active site. These seemingly insignificant changes in conformation are in fact, related to rate increases of many orders of magnitude (24, 25). Thus, it may be that very subtle dynamic behavior of biotin carboxylase is enough to generate the large increase in the rate of ATP hydrolysis upon the binding of biotin. Further studies will be required to determine this aspect of the mechanism.

In summary, the four active site residues of biotin carboxylase, Lys 116, Lys 159, His 209, and Glu 276, were shown to be involved in binding ATP. Furthermore, these four residues have also been found to be involved in catalysis, and their role in catalysis is to orient ATP in the correct conformation that allows for optimal catalysis. Finally, the results also suggest that the crystal structure of the mutant biotin carboxylase, E288K complexed with ATP, may not be a completely accurate depiction for the binding of ATP to the wild type form of biotin carboxylase.

## References

1. Wakil, S.J., Stopps, J.K., and Joshi, V.C. (1983) *Ann Rev Biochem* **52**, 537-579.
2. Knowles, J.R. (1989) *Ann Rev Biochem* **58**, 195-221.
3. Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J., and Lane, D.M. (1974) *J Biol Chem* **249**, 6633-6645.
4. Waldrop, G.L., Rayment, I., and Holden, H. (1994) *Biochemistry* **33**, 10249-10256
5. Artymiuk, P.J., Poirrette, A.R., Rice, D. W., and Willett, P. (1996) *Nat Struct Biol* **3**, 128-132.
6. Galperin, M.Y. and Koonin, E.V. (1997) *Protein Sci* **6**, 2639-2643.
7. Fan, C., Moews, P.C., Walsh, C.T., and Knox, J.R. (1994) *Science* **266**, 439-443.
8. Yamaguchi, H., Kato, H., Hata, Y., Nishioka, T., Kimura, A., Oda, J., and Katsube, Y. (1993) *J Mol Biol* **229**, 1083-1100.
9. Thoden, J.B., Holden, H.M., Wesenberg, G., Raushel, F.M., and Rayment, I. (1997) *Biochemistry* **36**, 6305-6316.
10. Kanai, S. and Toh, H. (1999) *J Mol Evol* **48**, 482-492.
11. Thoden, J.B., Kappock, T.J., Stubbe, J., and Holden, H.M. (1999) *Biochemistry* **38**, 15480-15492.
12. Thoden, J.B. Firestine, S., Nixon, A., Benkovic, S.J., and Holden, H.M. (2000) *Biochemistry* **39**, 8791-8802.
13. Blanchard, C.Z., Lee, Y.M., Frantom, P.A., and Waldrop, G.L. (1999) *Biochemistry* **38**, 3393-3400.
14. Thoden, J.B., Blanchard, C.Z., Holden, H.M., and Waldrop, G.L. (2000) *J Biol Chem* **275**, 16183-16190.
15. Ogita, T. and Knowles, J.R. (1988) *Biochemistry* **27**, 8028-8033.
16. Polakis, S.E., Guchhait, R.B., Zwergel, E.E., Lane, M.D., and Cooper, T.G. (1974) *J Biol Chem* **249**, 6657-6667.
17. Stapleton, M.A., Javid-Majd, F., Harmon, M.F., Hanks, B.A., Grahmann, J.L., Mullins, L.S., and Raushel, F.M. (1996) *Biochemistry* **35**, 14352-14361.

18. Shi, Y. and Walsh, C. T. (1995) *Biochemistry* **34**, 2768-2776.
19. Bruice, T.C. and Lightstone, F.C. (1999) *Acc Chem Res*, **32**, 127-136.
20. Bruice, T.C. and Benkovic, S.J. (2000) *Biochemistry* **39**, 6267-6274.
21. Blanchard, C.Z., Amspacher, D., Strongin, R., and Waldrop, G.L. (1999) *Biochem Biophys Res Commun* **266**, 466-471.
22. Sutcliffe, M.J. and Scrutton, N.S. (2000) *Trends Biochem Sci* **25** 405-408.
23. Kohen, A. and Klinman, J.H. (2000) *J Am Chem Sci* **122**, 10738-10739.
24. Koshland, D.E. (1998) *Nat Med* **4**, 1112-1114.
25. Koshland, D.E. (1994) *Agnew Chem Int Ed Engl* **33**, 2375-2378.
26. Levert, K.L., Lloyd, R.B., and Waldrop, G. L. (2000) *Biochemistry* **39**, 4122-4128.
27. Thoden, J.B., Wesenberg, G., Raushel, F.M., and Holden, H.M. (1999) *Biochemistry* **38**, 2347-2357.

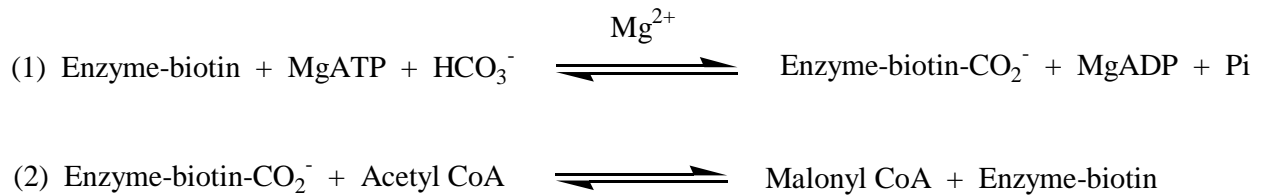
## CHAPTER 3

### KINETIC CHARACTERIZATION OF MUTATIONS FOUND IN PROPIONIC ACIDEMIA AND METHYLCROTONYL GLYCINURIA: EVIDENCE FOR NEGATIVE COOPERATIVITY IN BIOTIN CARBOXYLASE

#### Introduction

For over 20 years site-directed mutagenesis has been used to determine the roles of active site residues in enzyme-catalyzed reactions. In designing such experiments, one is invariably faced with the question of which residue to mutate and what to mutate it to. Residues targeted for mutagenesis are usually identified using three-dimensional structural information or sequence alignments with homologous proteins indicating which residues are highly conserved. Once residues for mutagenesis are identified, they are commonly replaced with an isosteric residue or alanine (i.e. alanine scanning mutagenesis) in order to minimize disruption of the protein structure. However, prior to the advent of site-directed mutagenesis, mutant human hemoglobins provided the only opportunity to study structure-function relationships in proteins. Many of these hemoglobinopathies involved amino acid replacements that would not normally be made using site-directed mutagenesis out of concern for disruption of the protein structure. Yet these mutations yielded important insight into how hemoglobin functioned (1). In this report, we take this classical approach to mutagenesis to examine the effect of three naturally occurring mutations found in biotin-dependent carboxylases on the catalytic mechanism of *E. coli* biotin carboxylase.

Biotin carboxylase is one component of the multifunctional enzyme acetyl-CoA carboxylase, which catalyzes the first committed step in long chain fatty acid biosynthesis (2). Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the following two-step reaction (Scheme 3.1).



Scheme 3.1

Biotin carboxylase catalyzes the first-half reaction, which is an ATP-dependent carboxylation of biotin to form carboxybiotin. Biotin is covalently attached to the biotin carboxyl carrier protein designated Enzyme-biotin in Scheme 3.1. The second-half reaction is catalyzed by carboxyltransferase, which involves the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to make malonyl-CoA. Animal acetyl-CoA carboxylase incorporates all three of these functions on a single polypeptide chain (3), while in the bacterial form each function is a separate protein (4).

There have been no documented naturally occurring mutations in acetyl-CoA carboxylase leading to an inborn error of metabolism. However, there are two related biotin-dependent carboxylases in which naturally occurring mutations have been detected. Propionyl-CoA carboxylase is involved in the catabolism of odd chain length fatty acids and branched chain amino acids and mutations in the human form of this enzyme result in the condition propionic acidemia (5). The enzyme 3-methylcrotonyl-CoA carboxylase is involved in the catabolism of leucine and mutations in the human form of this enzyme result in methylcrotonylglycinuria (6). Both propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase catalyze their respective reactions via a two-step reaction sequence similar to the reaction catalyzed by acetyl-CoA carboxylase (Scheme 3.1). In fact, the first half-reaction in Scheme 3.1, the carboxylation

of biotin, is identical for all three enzymes. That is, propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase have a biotin carboxylase domain homologous to the biotin carboxylase domain of acetyl-CoA carboxylase. Although the genes for both propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase have been cloned and several disease-causing missense mutations identified, characterization of the mutant proteins has been limited to specific activity measurements of fibroblast extracts (7). This is due to the lack of a system for structure-function studies of these two enzymes. In contrast, the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase provides an ideal model system to conduct a rigorous kinetic analysis of mutations found in propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase. In fact, ten active site mutants of *E. coli* biotin carboxylase have been characterized to date (8, 9, 10). Kinetic characterization of these naturally occurring mutants using *E. coli* biotin carboxylase as a model may lend insight into the structure and function of the enzyme.

The advantage of using *E. coli* biotin carboxylase for kinetic analysis is that the homodimer can be isolated and that it retains catalytic activity in the absence of the other two components (11). Moreover, biotin carboxylase is able to utilize free biotin as a substrate instead of biotin linked to the biotin carboxyl carrier protein, thereby simplifying kinetic analysis (11). The availability of structural information is perhaps the most significant feature of *E. coli* biotin carboxylase that makes it the paradigm for mechanistic studies of biotin-dependent enzymes. The three-dimensional structure of biotin carboxylase has been determined by x-ray crystallography and to date is the only structural model of a biotin-dependent carboxylase (12, 13). Based on this crystal structure, two mutations in the biotin carboxylase domain of human propionyl-CoA carboxylase, M204K and R374Q, were predicted to be in the active site (5, 14). According to sequence alignments of the human propionyl-CoA carboxylase and *E. coli* biotin

carboxylase, the homologous residues in the *E. coli* enzyme are Met 169 and Arg 338 (Figure 3.1). In patients with methylcrotonylglycinuria, only one mutation of 3-methylcrotonyl-CoA carboxylase, R385S, was predicted to lie in the active site of the biotin carboxylase domain (6). Interestingly, this residue also corresponds to Arg 338 of *E. coli* biotin carboxylase (Figure 3.1). In this report, these three mutations, M169K, R338Q, and R338S, were constructed in *E. coli* biotin carboxylase and the mutant enzymes were subjected to kinetic characterization. The results not only suggest a molecular basis for understanding how these mutations cause disease, but also provide the first kinetic evidence for cooperativity between the subunits of *E. coli* biotin carboxylase.

## **Materials and Methods**

Chemicals and Enzymes. <sup>14</sup>C-labeled sodium bicarbonate was purchased from Amersham Pharmacia Biotech and had a specific activity of 0.1 mCi/mmol. His Bind resin was purchased from Novagen. DEAE cellulose resin was manufactured by Whatman. Pyruvate kinase was obtained from Boehringer Mannheim. All other reagents were from Sigma or Aldrich.

Site-Directed Mutagenesis. Construction of biotin carboxylase mutants was performed by the PCR method of overlap extension as described by Blanchard *et al.* (8). All primers were synthesized by Life Technologies, Inc. Mutagenic internal primers for the M169K, R338Q, and R338S mutants were used to incorporate the desired mutation. The mutagenic primer sequences were as follows: M169K: 5'GCGCACTACGCGCTTACCGCGACCGCC3' and 5'GGCGGTCGCGGTAAGCGCGTAGTGCGC3'; R338Q:

						169								
						▼								
EcBC	163-175	G	G	G	G	R	G	M	R	V	V	R	G	D
HsPCC	198-210	G	G	G	G	K	G	M	R	I	A	W	D	D

								338						
								▼						
EcBC	332-344	G	H	A	V	E	C	R	I	N	A	E	D	P
HsPCC	368-380	G	W	A	V	E	C	R	V	Y	A	E	D	P
HsMCC	379-391	G	H	A	F	E	A	R	I	Y	A	E	D	P

**Figure 3.1.** Amino acid sequences of biotin-dependent carboxylases. The regions of *Homo sapiens* propionyl-CoA carboxylase (HsPCC) and *Homo sapiens* 3-methylcrotonyl-CoA carboxylase (HsMCC) containing residues homologous to Met 169 and Arg 338 of *E. coli* biotin carboxylase (EcBC) are shown. The figure was created using Alscript (25).

5'TTCGGCGTTGATTTGACATTCCACCGC3' and  
5'GCGGTGGAATGTCAAATCAACGCCGAA3'; R338S:  
5'TTCGGCGTTGATAGAACATTCCACCGC3' and  
5'GCGGTGGAATGTTCTATCAACGCCGAA3'. The entire gene of each mutant was  
sequenced to confirm that only the desired mutation was incorporated.

Overexpression and Purification of Biotin Carboxylase. The growth and overexpression of wild type and mutant biotin carboxylase was performed as described by Blanchard *et al.* (8). Biotin carboxylase was purified by nickel affinity chromatography using His-Tag columns as described elsewhere (8) and the eluate from the His Bind resin was dialyzed overnight against buffer A (10 mM NaCl, 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.1) and was then applied to a DEAE cellulose column. The column was washed with 14 volumes of buffer A. The flowthrough and wash volumes were pooled and the protein was precipitated by the addition of ammonium sulfate to 60% saturation. The precipitate was dissolved and dialyzed against 500 mM KCl, 10 mM HEPES, pH 7.0. The resulting solution was then concentrated by vacuum dialysis using a collodion bag apparatus. Protein concentrations were determined by the Bio-Rad protein assay with BSA as a standard.

Kinetic Assays. The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically by coupling the formation of ADP to pyruvate kinase and lactate dehydrogenase and monitoring the oxidation of NADH at 340 nm. Each assay was conducted in the presence of 0.5 mM phosphoenolpyruvate, 0.4 mM NADH, 23 units of pyruvate kinase, 35 units of lactate dehydrogenase, and 100 mM HEPES at pH 8. Since the K<sub>m</sub> for biotin is high (134 mM), the ionic strength was kept constant with KCl in assays where biotin was varied.

The rate of ATP synthesis from Mg-ADP and carbamyl phosphate was measured spectrophotometrically. The formation of ATP was coupled to hexokinase and glucose-6-phosphate dehydrogenase while the reduction of NADP was followed at 340 nm. Each assay contained 0.5 mM glucose, 0.4 mM NADP, 5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 100 mM KCl, and 100 mM HEPES at pH 8.

Initial velocities were measured using a Uvikon 810 spectrophotometer (Kontron Instruments). All reactions were initiated by the addition of enzyme. Kinetic parameters were calculated per active site using a molecular mass of 50 kDa for the biotin carboxylase monomer (biotin carboxylase exists as a homodimer). The temperature of the reactions was maintained at 25°C or 37°C by a circulating water bath. For assays conducted at 37°C, all reaction components were preincubated for six minutes at 37°C prior to mixing in quartz cuvettes. For each initial velocity measurement, the cuvette was preincubated in the spectrophotometer at 37°C for 10 minutes before addition of the reaction components.

To determine if there was a stoichiometric production of ADP and carboxybiotin, the amount of carboxybiotin produced by biotin carboxylase was determined using a <sup>14</sup>C fixation assay and compared to the production of ADP as described elsewhere (8). The reaction mixtures contained 20 mM ATP, 70 mM bicarbonate, 100 mM biotin, 50 mM MgCl<sub>2</sub>, and 100mM HEPES at pH 8 in a total volume of 0.5 ml.

Data Analysis. the  $K_m$  and  $V_{max}$  were determined by nonlinear regression analysis of the velocity versus [substrate] data to the Michaelis-Menten equation. For assays in which the double reciprocal plots appeared hyperbolic, the data were fitted to equation 3.1, where  $V_1$  and  $V_2$  are maximal velocities,  $K_1$  and  $K_2$  are Michaelis constants, and  $A$  is the substrate concentration:

$$v = V_1A/(K_1 + A) + V_2A/(K_2 + A) \quad (3.1)$$

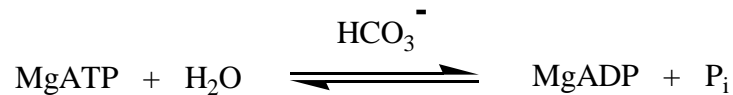
Hill coefficients ( $n_H$ ) were determined by nonlinear regression analysis of the velocity versus [bicarbonate] data to equation 3.2:

$$v = V_{\max} [A]^{n_H} / K' + [A]^{n_H} \quad (3.2)$$

where A is the substrate concentration and K' is a constant which depends upon the interaction between substrate binding sites and the intrinsic dissociation constant of the enzyme-substrate complex.

## Results

Bicarbonate-dependent ATPase Activity. The order of substrate binding to biotin carboxylase is ordered, with ATP binding first followed by bicarbonate and then biotin (15). However, in the absence of biotin, the enzyme also catalyzes a slow bicarbonate-dependent ATP hydrolysis, as shown in Scheme 3.2.



Scheme 3.2

This reaction involves the transfer of the phosphate group to the bicarbonate, forming carboxyphosphate, which quickly breaks down into carbon dioxide and inorganic phosphate (16). When ATP was varied at constant levels of bicarbonate, the kinetic parameters were determined for the three mutants and wild type. The  $V_{\max}$  values for the three mutants were only modestly different from the wild type enzyme; R338Q showed a two-fold increase in  $V_{\max}$  and R338S and M169K showed a two-fold decrease or less (Table 3.1). By contrast, the  $K_m$  was

**Table 3.1. Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction**

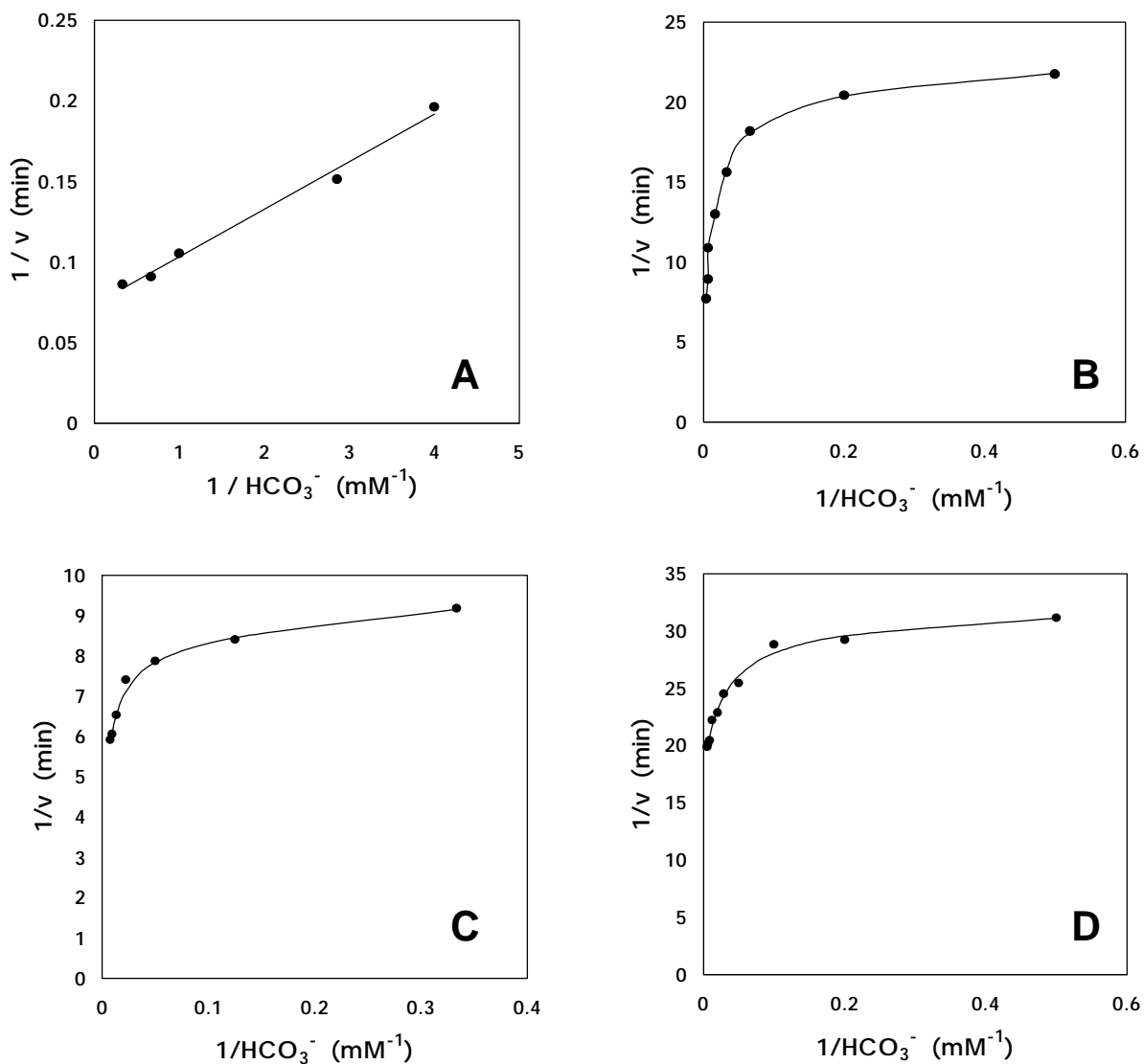
	$K_m$ ATP (mM)	$V_{max}$ ( $\text{min}^{-1}$ )
<b>WT<sup>a</sup></b>	$0.081 \pm 0.003$	$0.073 \pm 0.001$
<b>R338Q</b>	$4.4 \pm 0.3$	$0.16 \pm 0.01$
<b>R338S</b>	$5.1 \pm 0.05$	$0.045 \pm 0.002$
<b>M169K</b>	$0.26 \pm 0.02$	$0.033 \pm 0.001$

The kinetic parameters were determined by varying [ATP] at a constant level of bicarbonate. The concentration of bicarbonate was saturating for wild type, 30 mM for R338Q, 15 mM for R338S, and 10 mM for M169K. The errors on  $K_m$  and  $V_{max}$  were determined by nonlinear regression analysis. <sup>a</sup> Data taken from Ref. 8.

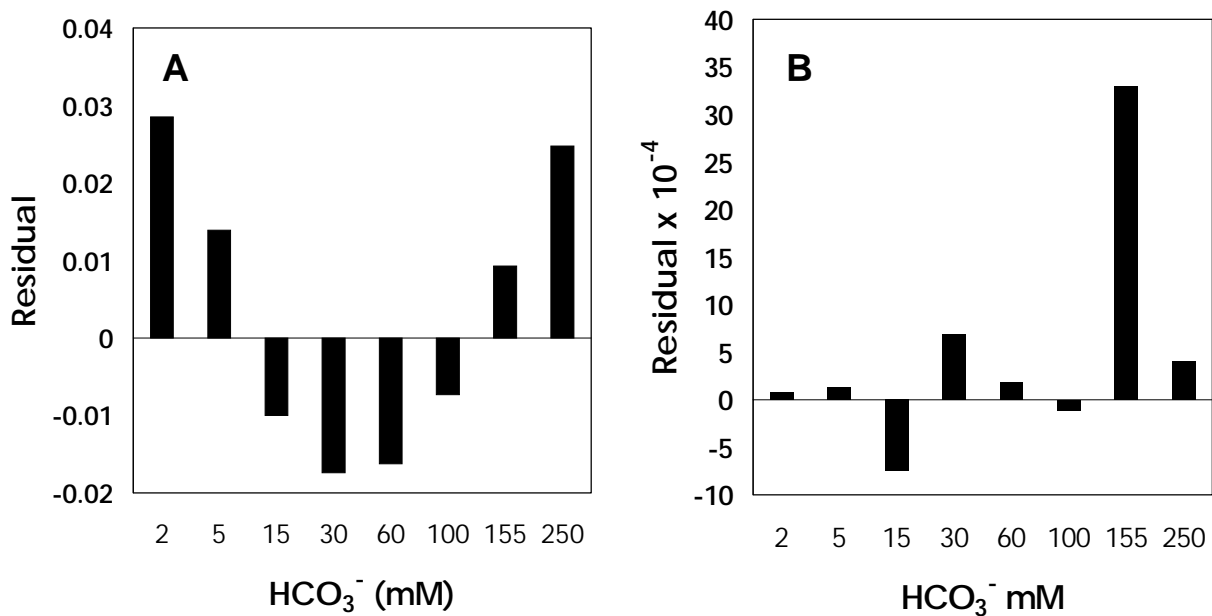
strikingly affected by the R338Q and R338S mutations. Both  $K_m$  values were elevated by at least 50-fold compared to wild type, suggesting the mutations have affected the binding affinity of ATP to the enzyme. The  $K_m$  for the M169K mutant was only slightly higher (about three-fold) than wild type.

Interestingly, when ATP was held constant and bicarbonate varied, the double reciprocal plots were hyperbolic for all three mutants (Figure 3.2). For all three mutants there was a significant increase in the velocity at high concentrations of substrate, suggesting substrate activation with respect to bicarbonate. Substrate activation, also known as negative cooperativity, was confirmed when the data for the three mutants were fitted to the Michaelis-Menten equation and the residuals were nonrandom. By contrast, fitting the data to the equation for negative cooperativity (Equation 3.2) revealed the residuals to be random. A representative example of this type of analysis is shown in Figure 3.3 for the M169K mutant. The fitting to equation 1 also revealed that the  $K_{m2}$  for each of the mutants was greater than 30 mM. For this reason, the  $K_m$  values for ATP shown in Table 3.1 are apparent  $K_m$  values because saturating levels of bicarbonate could not be achieved.

Particularly intriguing is the fact that the degree of negative cooperativity by bicarbonate for the three mutants is dependent upon the concentration of biotin. To illustrate this trend, the Hill coefficients ( $n_H$ ) under different concentrations of biotin are listed in Table 3.2. In the presence of 60 mM biotin, M169K showed a slight 23% increase in  $n_H$  relative to the reaction without biotin. The R338S and R338Q mutants displayed a more dramatic increase in  $n_H$  (3-fold and 5-fold, respectively) with 60 mM biotin as opposed to 16 mM biotin. These trends indicate that biotin decreases the negative cooperativity of the three mutants, particularly for the two Arg



**Figure 3.2.** Double reciprocal plots for the bicarbonate-dependent ATPase reaction. (A). Bicarbonate was varied at a constant saturating level of ATP in the presence of wild type biotin carboxylase. (B). Bicarbonate was varied at a constant saturating level of ATP in the presence of M169K. For R338S (C) and R338Q (D), bicarbonate was varied at 20 mM ATP and 16 mM biotin. Data points are experimental velocities and the line is derived from the best fit of the data to the Michaelis-Menten equation for wild type and equation 3.1 for the three mutants.



**Figure 3.3.** Residuals for the M169K mutant. Plots show the observed velocities minus the calculated velocities for data for the M169K mutant fitted to the Michaelis-Menten equation (A) and equation 3.1 (B).

**Table 3.2. Hill Coefficients for the three mutants of biotin carboxylase**

	<b>[Biotin]</b>	$n_H$
<b>R338Q</b>	16 mM	$0.17 \pm 0.03$
	60 mM	$0.79 \pm 0.07$
<b>R338S</b>	16 mM	$0.20 \pm 0.03$
	60 mM	$0.58 \pm 0.05$
<b>M169K</b>	0 mM	$0.29 \pm 0.02$
	60 mM	$0.38 \pm 0.04$

The Hill Coefficients ( $n_H$ ) were determined by fitting data from the bicarbonate-dependent ATPase assays to Equation 3.2. The error on  $n_H$  was derived from nonlinear regression analysis.

338 mutants. Assaying the two Arg 338 mutants in the absence of biotin proved difficult because the enzymes displayed almost no change in rate when bicarbonate was varied.

Biotin-dependent ATPase Activity. The rate of ATP hydrolysis is increased 1100-fold in the presence of biotin for the wild-type enzyme (8). However, when the hydrolysis of ATP was assayed in the presence of biotin for the three mutants, the most notable result was that the  $V_{\max}$  of R338S and R338Q was 700-fold and 100-fold lower than wild type, respectively (Table 3.3). The  $V_{\max}$  was also reduced in the M169K mutant, but only by about 10-fold. The  $K_m$  for biotin for R338Q was virtually unchanged from that of wild type. The R338S and M169K mutants, however, showed a 5-fold and 2.5-fold reduction in apparent  $K_m$ , respectively. Although the latter two mutants seem to bind biotin with slightly greater affinity than wild type, it is important to note that the catalytic efficiency ( $V/K$ ) is 5-fold lower for M169K and 140-fold lower for R338S. Again, the  $K_m$  for the three mutants reported here are apparent  $K_m$  values because it was not possible to saturate the enzymes with bicarbonate.

Although the assay for the ATPase activity measures the rate of production of ADP using a continuous assay, the production of carboxybiotin cannot be followed. Thus, the assay cannot detect whether ATP hydrolysis is uncoupled from the production of carboxybiotin in the mutants. However, carboxybiotin can be quantified in order to demonstrate whether it is produced at a 1:1 stoichiometry with ADP. Wild type biotin carboxylase and M169K both exhibited a normal 1:1 ratio of ADP to carboxybiotin production (Table 3.4). Unexpectedly, however, R338S and R338Q revealed ratios of 3.0 and 3.8, respectively. This conspicuous perturbation in the stoichiometry suggests that for the two Arg 338 mutants, the carboxylation of biotin is uncoupled from ATP hydrolysis. Of the ten site-directed mutants of biotin carboxylase characterized to date, only one other, K238Q, has exhibited such an uncoupling (9).

**Table 3.3. Kinetic parameters for the Biotin-Dependent ATPase Reaction**

	<b>K<sub>m</sub> Biotin</b> (mM)	<b>V<sub>max</sub></b> (min <sup>-1</sup> )	<b>V/K</b> (mM <sup>-1</sup> min <sup>-1</sup> )
<b>WT<sup>a</sup></b>	134 ± 14	79 ± 3	0.59 ± 0.08
<b>R338Q</b>	143 ± 21	0.74 ± 0.05	0.0052 ± 0.0004
<b>R338S</b>	25 ± 2	0.10 ± 0.01	0.0041 ± 0.0003
<b>M169K</b>	56 ± 6	6.7 ± 0.3	0.12 ± 0.01

The kinetic parameters were obtained by varying [biotin] at constant levels of ATP and bicarbonate. For all mutants, bicarbonate was held at the same levels as in Table 3.1. ATP concentration was saturating for M169K and wild type. For R338Q and R338S, ATP was held at the highest concentration that did not produce substrate inhibition. The errors on K<sub>m</sub>, V<sub>max</sub>, and V/K were determined by nonlinear regression analysis.

**Table 3.4. Ratio of ADP to Carboxybiotin Production**

<b>WT<sup>a</sup></b>	$0.96 \pm 0.18$
<b>R338Q</b>	$3.8 \pm 0.1$
<b>R338S</b>	$3.0 \pm 0.1$
<b>M169K</b>	$1.0 \pm 0.1$

Quantities of ADP and carboxybiotin formed over a 1-hour period were determined as described in Materials and Methods. The error was derived from the standard deviation of the mean of at least two separate experiments. <sup>a</sup> Data taken from Ref. 10.

ATP Synthesis Activity. *E. coli* biotin carboxylase is known to catalyze the formation of ATP from ADP and carbamyl phosphate, as shown in Scheme 3.3.



Scheme 3.3

The carbamate molecule rapidly decomposes to carbon dioxide and ammonia. This reaction mimics the reverse of the physiological reaction, with carbamyl phosphate acting as a carboxyphosphate analog. Note that the ATP synthesis reaction does not include biotin, although biotin does stimulate the rate of ADP phosphorylation (8). The three mutations do not appear to have markedly altered the kinetics of the ATP synthesis reaction (Table 3.5). The only dissimilarity to wild type kinetics was exhibited by the two Arg 338 mutants. R338S had a  $V_{\max}$  reduced by 2-fold and a  $K_m$  ADP increased by two-fold. R338Q displayed a three-fold increase in  $K_m$  ADP, and two-fold decrease in  $K_m$  CbmP, and a 5-fold increase in  $V_{\max}$ .

The degree of stimulation of the phosphoryl transfer by biotin was also compared for wild type and the three mutants. The rate of ATP synthesis was examined with and without 60 mM biotin (Table 3.6). Compared to wild type, the stimulation factor was slightly reduced for R338S and R338Q (two-fold or less) and increased by two-fold for M169K.

Effect of Temperature on Activity. Clearly, it seems that the diseases caused by the three homologous mutations of human biotin-dependent carboxylases are attributable, at least in part, to their decreased activity. The impaired  $V_{\max}$  of the biotin-dependent ATPase reaction by the homologous biotin carboxylase mutants corroborates this notion. However, since the assays on the biotin carboxylase mutants were carried out at 25° C, denaturation of the protein structure at body temperature (37° C) could not be ruled out. Thus, the biotin-dependent ATPase reaction

**Table 3.5. Kinetic Parameters for the ATP Synthesis Reaction**

	<b>K<sub>m</sub> CbmP</b> (mM)	<b>K<sub>m</sub> ADP</b> (mM)	<b>V<sub>max</sub></b> (min <sup>-1</sup> )
<b>WT<sup>a</sup></b>	4.8 ± 0.2	0.19 ± 0.01	0.31 ± 0.01
<b>R338Q</b>	2.4 ± 0.2	0.65 ± 0.05	1.47 ± 0.04
<b>R338S</b>	6.9 ± 0.9	0.39 ± 0.04	0.14 ± 0.01
<b>M169K</b>	3.6 ± 0.6	0.14 ± 0.01	0.22 ± 0.01

The kinetic parameters were determined by varying [CbmP] at constant saturating levels of ADP or by varying [ADP] at constant saturating levels of CbmP. The standard errors on K<sub>m</sub> and V<sub>max</sub> were determined by nonlinear regression analysis. <sup>a</sup> Data taken from Ref. 9.

**Table 3.6. Stimulation of ATP Synthesis by Biotin**

<b>Stimulation Factor</b>	
<b>WT<sup>a</sup></b>	25
<b>R338Q</b>	18
<b>R338S</b>	12
<b>M169K</b>	53

The initial velocity of the ATP synthesis reaction was measured both in the presence and absence of 60 mM biotin. All reactions contained saturating levels of CbmP, ADP, and magnesium. The stimulation factor is the ratio of the rate with biotin to the rate without biotin. <sup>a</sup> Data taken from Ref. 10.

was carried out at 37° C for R338Q, R338S, and M169K. Compared to the data collected at 25° C, the  $V_{\max}$  at 37° C was two-fold higher for both wild type and R338Q. R338S displayed a three-fold increase in  $V_{\max}$  at 37° C, while M169K displayed a seven-fold increase.

Since the activity of the three mutants increased at 37° C, it can be surmised that the overall protein structure was not damaged by the physiological temperature, and that the kinetics observed for the mutants are representative of those *in vivo*. This assertion is further confirmed by Western blot analysis of fibroblast extracts from patients carrying the mutations M204K and R385S. Biotin carboxylase subunits of propionyl-CoA carboxylase were detected in the patient fibroblasts with the M204K mutation (14). Biotin carboxylase subunits of 3-methylcrotonyl-CoA carboxylase were also detected in patient fibroblasts carrying the R385S mutation (6). The presence of these proteins also argues against any intrinsic instability and subsequent degradation of the enzyme due to the mutations.

## **Discussion**

In this report, we used naturally occurring mutations of biotin-dependent carboxylases to identify active site residues of *E. coli* biotin carboxylase for mutational analysis. The fact that these mutations result in metabolic diseases in humans suggests that the homologous mutants in *E. coli* biotin carboxylase will have altered kinetics that provide insight into the mechanism of the enzyme as well as determine the molecular basis of the diseases. Indeed, these naturally occurring mutants revealed two important observations concerning the mechanism of catalysis.

The most striking result was that all three mutants of *E. coli* biotin carboxylase exhibited negative cooperativity with respect to the substrate bicarbonate. This is the first evidence that one of the natural substrates exhibits cooperative behavior in *E. coli* biotin carboxylase and substantiates previous work suggesting that the two subunits communicate (8, 17). The first

evidence for communication between the two subunits of *E. coli* biotin carboxylase was that an active site mutant, N290A, exhibited negative cooperativity with respect to carbamyl phosphate in a slow non-physiological reaction where the phosphate group is transferred to ADP to make ATP (8). Interestingly, carbamyl phosphate is an analog of the reaction intermediate carboxyphosphate, which is the phosphorylated form of bicarbonate. A more compelling case for communication between the subunits involved making hybrid dimers of biotin carboxylase where one subunit had a wild-type active site while the other subunit contained an active site mutation known to significantly decrease the activity of the enzyme (17). The active sites of the enzyme are not at the interface between the two subunits (12). That is, the active sites are not composed of residues from both subunits (also referred to as a shared active site). Kinetic analyses of four hybrid dimers had  $V_{\max}$  values that were 0.4 to 3.6% of the  $V_{\max}$  value for dimers composed of two wild type subunits. Thus, the two subunits of biotin carboxylase do not function independently. If the two subunits acted independently, the  $V_{\max}$  values of the hybrids would have been about half of the  $V_{\max}$  of the wild type enzyme. All four mutations exhibited a dominant negative effect on the function of the wild type active site, suggesting there is communication between the subunits. The observation of negative cooperativity in the N290A, R338S, R338Q, and M169K mutants is consistent with the notion of communication between the subunits and substantiates the possibility that the binding of substrate to one subunit affects the kinetics of the other subunit.

The fact that a point mutation results in cooperative behavior suggests that multisubunit proteins are teetering between cooperative and noncooperative behavior. There are numerous examples of single amino acid substitutions bestowing cooperativity or abolishing cooperativity in proteins. Examples are found in hemoglobin (1), aspartate transcarbamylase (18), pyruvate

kinase (19) and the aspartate receptor (20). Given the precarious balance between cooperative and noncooperative behavior, is the negative cooperativity observed here simply an artifact of the mutations or does it provide any insight into the function of the enzyme? It is tempting to speculate that the mutant forms of biotin carboxylase that exhibit negative cooperativity represent evolutionary relics. This notion is supported by noting that the composition of the early atmosphere contained high levels of carbon dioxide (21). Negative cooperativity was detected only with respect to bicarbonate, not ATP or biotin. Negative cooperativity with respect to bicarbonate could have conferred an evolutionary advantage to the organism by decreasing the sensitivity of the enzyme to bicarbonate concentration. This would allow the enzyme to be active over a broader range of bicarbonate concentrations than would be allowed by Michaelis-Menten kinetics (22). As the level of CO<sub>2</sub> in the environment stabilized, negative cooperativity of biotin carboxylase would have ceased to confer any advantage for the organism. Thus the gene coding for the enzyme would mutate to create an enzyme obeying Michaelis-Menten kinetics.

In addition to negative cooperativity, the two Arg 338 mutants (R338Q and R338S) revealed that arginine plays a significant role in carboxyl transfer to biotin. The 3:1 ratio of ADP produced to carboxybiotin produced indicates that cleavage of ATP is uncoupled from carboxyl transfer to biotin. What function could Arg 338 serve in the chemistry of carboxyl transfer? One possibility would be to abstract the N1' proton from biotin to allow for carboxylation. However, finding an active site residue that acts as a catalytic base in biotin carboxylase has been unsuccessful to date despite the mutation of ten active site amino acids. Furthermore, there is virtually no precedent for an arginine acting as a general base in enzymic reactions. An alternate explanation of the role of Arg 338 begins with the work of Levert *et al.* who implicated Lys 238

as an essential residue in the production of carboxybiotin (9). Like the Arg 338 mutants, mutation of Lys 238 resulted in uncoupling of ATP hydrolysis and biotin carboxylation. It is interesting to note that of all the site-directed mutants of biotin carboxylase that have been characterized, only mutations of Lys 238 and Arg 338 have resulted in uncoupling of ATP hydrolysis and biotin carboxylation. Lys 238 acting as a catalytic base was ruled out because the pKa value was found to be greater than 9.5. The  $K_m$  for ATP for K238Q was 50-fold higher than that of wild type, while the  $K_m$  for ADP was only 5-fold higher. Similarly, R338Q and R338S displayed a 50-fold increase in the  $K_m$  for ATP along with a modest 3-fold increase in the  $K_m$  for ADP. Given the similarity in kinetics between K238Q and the two Arg 338 mutants, it is likely that their catalytic roles are similar. Since Levert *et al.* concluded that Lys 238 interacted with the gamma phosphate of ATP, it may be inferred that Arg 338 also interacts with the gamma phosphate. Levert *et al.* went on to conclude that if Lys 238 binds to the gamma phosphate group of ATP and in turn binds the phosphate group of carboxyphosphate, then Lys 238 may help orient the phosphate group to act as a base for extraction of the proton from the 1' nitrogen of biotin. Thus, Arg 338 may act in concert with Lys 238 in the alignment of the phosphate group of carboxyphosphate to carry out substrate-assisted catalysis, as illustrated in Figure 3.4. Although this model seems inconsistent with the observation that the  $K_m$  for CbmP (a carboxyphosphate analog) for the two Arg 338 mutants was not significantly different than that of wild type, it should be noted that CbmP is not the physiological substrate. In fact, the amide nitrogen of CbmP can act as a hydrogen bond donor, whereas the carboxyl oxygen of carboxyphosphate can act as a hydrogen bond acceptor. The fact that CbmP can have different hydrogen bonding properties may cause it to be positioned differently than carboxyphosphate in

the active site. Thus, although CbmP is an analog of a physiological intermediate, it may not exactly mimic the binding of carboxyphosphate.

Does the kinetic analysis of the mutants found in patients with propionic acidemia and methylcrotonylglycinuria correlate with the clinical phenotype? The patient with the mutation M204K in propionyl-CoA carboxylase exhibited normal growth and development when treated with a protein restricted diet (23). This mild clinical phenotype is consistent with the observation that the overall function of the homologous mutation in *E. coli* biotin carboxylase (M169K) did not severely cripple the enzyme. The mutant enzyme had a maximal velocity 10% of the wild-type value and exhibited negative cooperativity with respect to bicarbonate. However, all other aspects of the kinetics of the M169K mutant were close to wild type, and most importantly, the ratio of ADP to carboxybiotin production was 1:1. This is in contrast to the R338S mutant, which showed a decreased production of carboxybiotin relative to ADP and a maximal velocity that was 100-fold less than the wild type value. The fact that the R338S mutation resulted in a defective *E. coli* biotin carboxylase is consistent with the severe phenotype exhibited by the patient carrying the homologous mutation (R385S) in 3-methylcrotonyl-CoA carboxylase (6, 24). The clinical phenotype of the patient with the mutation (R374Q) in propionyl-CoA carboxylase was not available.

It is important to note that although the M169K and R338S mutations both reduced the  $V_{\max}$ , neither mutation completely abolished the carboxylation of biotin. Likewise, the residual activity of the homologous mutant carboxylases in both patients was sufficient to allow compensation for the deficiency through a modified diet (23, 24). By decreasing the flux of metabolites through the amino acid catabolic pathway, the buildup of toxic intermediates can be reduced. However, the severity of the two phenotypes was quite different, reflecting the

importance of the individual roles of the two mutated residues. Despite the protein restricted diet, the patient carrying the R385S mutation sustained irreversible brain damage and delayed development. In contrast, the patient carrying the M204K mutation had normal growth and development. Thus, the classical approach of studying naturally occurring, disease-causing mutations in proteins allows genotype-phenotype correlations to be made.

## References

1. Perutz, M. F. (1990) *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*, Cambridge University Press, Cambridge, England.
2. Wakil, S. J., Stopps, J. K., and Joshi, V. C. (1983) *Annu. Rev. Biochem.* **52**, 537-579
3. Tanabe, T., Wada, K., Okazaki, T., and Numa, S. (1975) *Eur. J. Biochem.* **57**, 15-24
4. Cronan, J. E. and Waldrop, G. L. (2002) *Prog. Lipid Res.* **41**, 407-435
5. Ugarte, M., Pérez-Cerdá, C., Rodríguez-Pombo, P., Desviat L. R., Pérez, B., Richard, E., Muro, S., Campeau, E., Ohura, T., and Gravel, R.A. (1999) *Hum. Mutat.* **14**, 275-282
6. Baumgartner, M. R., Almashanu, S., Suormala, T., Obie, C., Cole, R. N., Packman, S., Baumgartner, E. R., and Valle, D. (2001) *J. Clin. Invest.* **107**, 495-504
7. Clavero, S., Martínez, A., Pérez, B., Pérez-Cerdá, C., Ugarte, M., and Desviat, L. R. (2002) *Biochim. Biophys. Acta.* **1588**, 119-125
8. Blanchard, C. Z., Lee, Y. M., Frantom, P. A., and Waldrop, G. L. (1999) *Biochemistry* **38**, 3393-3400
9. Levert, K. L., Lloyd, R. B., and Waldrop, G. L. (2000) *Biochemistry* **39**, 4122-4128
10. Sloane, V., Blanchard, C. Z., Guillot, F., and Waldrop, G. L. (2001) *J. Biol. Chem.* **276**, 24991-24996
11. Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., and Lane, D. M. (1974) *J. Biol. Chem.* **249**, 6633-6645
12. Waldrop, G. L., Rayment, I., and Holden, H. (1994) *Biochemistry* **33**, 10249-10256
13. Thoden, J. B., Blanchard, C. Z., Holden, H., and Waldrop, G. L. (2000) *J. Biol. Chem.* **275**, 16183-16190

14. Campeau, E., Dupuis, L., León-Del-Río, A., and Gravel, R. (1999) *Mol. Genet. Metab.* **67**, 11-22
15. Blanchard, C. Z., Amspacher, D., Strongin, R., and Waldrop, G. L. (1999) *Biochem. Biophys. Res. Commun.* **266**, 466-471
16. Climent, I., and Rubio, V. (1986) *Arch. Biochem. Biophys.* **251**, 465-470.
17. Janiyani, K., Bordelon, T., Waldrop, G. L., and Cronan, J. E., Jr. (2001) *J. Biol. Chem.* **276**, 29864-29870
18. Eisenstein, E., Markby, D. W., and Schachman, H. K. (1990) *Biochemistry* **29**, 3724-3731
19. Ikeda, Y., Tanaka, T., and Noguchi, T. (1997) *J. Biol. Chem.* **272**, 20495-20501
20. Kolodziej, A. F., Tan, T., and Koshland, D. E., Jr. (1996) *Biochemistry* **35**, 14782-14792
21. Lazcano, A., and Miller, S. L. (1996) *Cell* **85**, 793-798
22. Koshland, D. E., Jr. and Hamadani, K. (2002) *J. Biol. Chem.* **277**, 46841-46844
23. Brandt, I. K., Hsia, Y. E., Clement, D. H., and Provence, S. A. (1974) *Pediatrics* **53**, 391-395
24. Steen, C., Baumgartner, E. R., Duran, M., Lehnert, W., Suormala, T., Fingerhut, R., Stehn, M., and Kohlschütter, A. (1999) *Eur. J. Pediatr.* **158**, 730-733
25. Barton, G. J. (1993) *Protein Eng.* **6**, 37-40.

## CHAPTER 4

### CONCLUSIONS

The studies presented in this dissertation take advantage of the availability of 3-dimensional structural information on *E. coli* biotin carboxylase, as well as the fact that the enzyme is cloned, overexpressed and purified successfully. These features constitute an excellent system for the construction and characterization of site-directed mutants. Ample amounts of mutant protein can be obtained, ensuring unambiguous kinetic data, while structural data can be used to aid kinetic interpretation. For these reasons, *E. coli* biotin carboxylase provides an ideal model system for mechanistic studies applicable to all biotin-dependent carboxylases.

In the first study, the question of how four highly conserved active site residues of biotin carboxylase can play a role in catalysis was addressed. The residues Lys 116, Lys 159, His 209, and Glu 276 were of interest because they were among the few residues with a high degree of conservation in the ATP-grasp superfamily. Fortunately, crystal structures are available for several enzymes of the superfamily, and the highly conserved residues can be visualized along with their putative interactions with bound substrate (or substrate analogs). In this way, specific roles for the homologous residues in *E. coli* biotin carboxylase can be inferred. By examining the crystal structures of D-ala D-ala ligase, glutathione synthetase, carbamyl phosphate synthetase, glycylamide ribonucleotide transformylase, and *N*<sup>5</sup>-carboxyaminoimidazole ribonucleotide transformylase, the four residues were all postulated to be involved in contacts with various moieties of the substrate ATP. Later however, when the crystal structure of biotin carboxylase was solved with ATP in the active site, the postulated interactions by Lys 159, His

209, and Glu 276 were brought into question; they were not consistent with the interactions suggested by other ATP-grasp enzymes. However, in light of the fact that the crystallized protein contained a mutation of an essential active site residue (to prevent hydrolysis of the ATP ligand), the discrepancies may have been artifactual. To verify this possibility, and to explore the roles of the four residues in the active site of biotin carboxylase, the mutations K116Q, K159Q, H209A, and E276Q were constructed and analyzed by steady-state kinetics. Not surprisingly, the  $K_m$  values for ATP for all four mutants were elevated 40- to 90-fold relative to wild type. This suggests that the four residues are indeed involved in binding ATP. As such, the liganded crystal structure of the mutant biotin carboxylase proved not to be perfectly representative of the wild type enzyme.

Another conspicuous difference between the mutant and wild type kinetics was that the  $V_{max}$  for the biotin-dependent ATPase activity was reduced between 30- and 260-fold. This suggests that the four residues have significantly reduced substrate-induced synergism by biotin, a perturbation caused by misalignment of ATP in the active site. In the wild type enzyme, the binding of biotin causes ATP to bind more productively in the active site and an increase in  $V_{max}$  (*i.e.*, substrate-induced synergism) is observed. On the other hand, a mutation of an important residue might lead to a shift in the active site geometry that increases the number of nonproductive binding modes of ATP. This manifests as a reduced rate of catalysis (*i.e.*, no substrate synergism).

In the second site-directed mutagenesis study, three mutations were selected for study based on the fact that the homologous mutation in human propionyl-CoA carboxylase or 3-methylcrotonyl-CoA carboxylase causes a metabolic disease resulting from deficient amino acid catabolism. The mutations M204K and R374Q of human propionyl-CoA carboxylase cause

propionic acidemia, while R385S of human 3-methylcrotonyl-CoA carboxylase causes methylcrotonylglycinuria. The corresponding mutants in *E. coli* biotin carboxylase are M169K, R338Q, and R338S.

Examination of the kinetics of these three mutants revealed that the production of ADP and carboxybiotin was not stoichiometric in R338Q and R338S. The ratio of ATP to carboxybiotin was approximately 3:1 for both mutants, suggesting sluggish production of carboxybiotin. To date, only one other residue in biotin carboxylase, Lys 238, has been proven to play a role in the stoichiometry of the biotin-dependent ATPase reaction. Thus it was postulated that, since neither residue is likely to act as a catalytic base to deprotonate biotin, both residues may be involved in positioning the carboxyphosphate intermediate for optimal carboxyl transfer. This model is consistent with the fact that the  $K_m$  for ATP for K238Q, R338Q, and R338S, was elevated but the  $K_m$  for ADP was not. These results imply an interaction with the  $\gamma$ -phosphate of ATP, which contributes to the carboxyphosphate intermediate.

The most intriguing kinetic result, and one which was obtained for all three mutants, was the observation of negative cooperativity with respect to bicarbonate. The fact that negatively cooperative kinetics is observed in a naturally-occurring mutant may not be purely coincidental. There are several other known examples of single amino acid substitutions giving rise to cooperative behavior. Furthermore, it seems likely that the mutations in this study represent evolutionary relics. It has been speculated that there were high levels of  $\text{CO}_2$  in the prebiotic atmosphere, which would imply high concentrations (and perhaps large fluctuations) of bicarbonate in solution. Thus, an enzyme responsive to a broad range of substrate concentrations, which could be accomplished by negatively cooperative kinetics, could be beneficial to an organism.

With respect to the diseases, the kinetic data correlates with the known phenotypes of the patients carrying the homologous mutation. The phenotype of the patient carrying the R385S mutation was severe, early onset neurological involvement with severe developmental delay. This is presumably due to a low level of residual activity of 3-methylcrotonyl-CoA carboxylase, which is in good agreement with the drastically (100-fold) reduced  $V_{\max}$  of the biotin-dependent ATPase activity and the sluggish carboxylation of biotin exhibited by the R338S mutant of biotin carboxylase. On the other hand, the  $V_{\max}$  for M169K was reduced by only 10-fold, implying a higher level of residual activity *in vivo*. Indeed, the phenotype of the patient carrying the M204K mutation was mild; normal growth and development was achieved in response to a protein-restricted diet.

The use of the well-established *E. coli* biotin carboxylase model system in the two studies of this dissertation have contributed significantly to our understanding of the catalytic mechanism of biotin carboxylase. In both studies, the data obtained from the structural and kinetic study of biotin carboxylase was shown to be applicable to other ATP-grasp enzymes (including the other biotin-dependent carboxylases), and vice versa. Furthermore, the kinetic examination of naturally-occurring, disease-causing mutations allows a molecular basis for disease to be established.

**APPENDIX**  
**LETTER OF PERMISSION**

Sent by: jcuthbert@asbmb.faseb.org

To: vsloan1@lsu.edu

cc:

Subject: Permissions

Dear Dr. Sloane:

Please note your recent request for copyright permission has been granted for the following:

Vol	Page	Year
276	24991-24996	2001

Please note your original will follow by mail only if requested. For future requests, we can be contacted by e-mail at jbc@asbmb.faseb.org. If you have any questions, please contact us @301-530-7150.

Sincerely yours,

PERMISSION GRANTED

**Joan Cuthbert**

---

for the copyright owner  
THE AMERICAN SOCIETY FOR BIOCHEMISTRY  
AND MOLECULAR BIOLOGY

## VITA

Valerie Melissa Sloane was born in Lafayette, Louisiana, on November 4, 1972. She attended high school at Episcopal School of Acadiana in Cade, Louisiana, and graduated in 1990. She then attended the University of Southwestern Louisiana (now known as the University of Louisiana at Lafayette), where she was awarded a Bachelor of Science Degree in biology-chemistry in 1995. After spending two years working at Sherry Laboratories in Lafayette, she enrolled in the Department of Biological Sciences at Louisiana State University, where she investigated the mechanism of the enzyme biotin carboxylase in the laboratory of Dr. Grover Waldrop. She will complete the requirements for the Doctor of Philosophy Degree in biochemistry in May, 2004. In February of 2004, she plans to move to Atlanta, Georgia, and work for Dr. Andrew Neish in the Department of Pathology at Emory University.