Site-directed Mutagenesis of ATP Binding Residues of Biotin Carboxylase

INSIGHT INTO THE MECHANISM OF CATALYSIS*

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Acetyl-CoA carboxylase catalyzes the first committed step in fatty acid synthesis in all plants, animals, and bacteria. The Escherichia coli form is a multimeric protein complex consisting of three distinct and separate components: biotin carboxylase, carboxyltransferase, and the biotin carboxyl carrier protein. The biotin carboxylase component catalyzes the ATP-dependent carboxylation of biotin using bicarbonate as the carboxylate source and has a distinct architecture that is characteristic of the ATP-grasp superfamily of enzymes. Included in this superfamily are d-Ala d-Ala ligase, glutathione synthetase, carbamyl phosphate synthetase, N⁵-carboxyaminoimidazole ribonucleotide synthetase, and glycaminide ribonucleotide transformylase, all of which have known three-dimensional structures and contain a number of highly conserved residues between them. Four of these residues of biotin carboxylase, Lys-116, Lys-159, His-209, and Glu-276, were selected for site-directed mutagenesis studies based on their structural homology with conserved residues of other ATP-grasp enzymes. These mutants were subjected to kinetic analysis to characterize their roles in substrate binding and catalysis. In all four mutants, the $K_m$ value for ATP was significantly increased, implicating these residues in the binding of ATP. This result is consistent with the crystal structures of several other ATP-grasp enzymes, which have shown specific interactions between the corresponding homologous residues and co-crystallized ADP or nucleotide analogs. In addition, the maximal velocity of the reaction was significantly reduced (between 30- and 260-fold) in the 4 mutants relative to wild type. The data suggest that the mutations have misaligned the reactants for optimal catalysis.

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 two-step reaction mechanism (1).

\[
\begin{align*}
\text{Enzyme-biotin + MgATP + HCO}_3^- &\rightarrow \text{Enzyme-biotin-CO}_2^- + MgADP + P_i \\
\text{Enzyme-biotin-CO}_2^- + Acetyl-CoA &\rightarrow \text{Malonyl-CoA + Enzyme-biotin}
\end{align*}
\]

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).

Two years after the solution of the crystal structure, Artyumiuk et al. (5) observed that biotin carboxylase had a strong structural homology to glutathione synthetase and d-Ala d-Ala ligase. 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, Artyumiuk et al. (5) 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 Artyumiuk et al. (5), the three-dimensional structure of carba-
mutyal phosphatase 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 structures of two of these enzymes, \( \text{N}^5\text{-carboxyaminoimidazole ribonucleotide synthetase} \) (11) and glycaminide 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 definitely important for catalysis (13). In fact, mutation of Glu-288 and Asn-290 suggested that the two residues interact with ATP, whereas the structures of the other ATP-grasp enzymes were found to interact with ATP. However, there were 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, whereas the structures of the other ATP-grasp enzymes indicated that 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 \(^1^4\)C was from Amersham Pharmacia Biotech and had a specific activity of 0.1 mCi/mmol. His binding resin was from Novagen. His tag on biotin carboxylase was carried out by the PCR method of overlap extension as previously described (13). 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 primers used to make each site-directed mutant can be found in Table I. 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. The formation of ATP was coupled to hexokinase and glucose-6-phosphate dehydrogenase, with the production of 

\[ \text{MgATP} + \text{H}_{2}\text{O} \rightarrow \text{MgADP} + \text{Pi} \]

**Reaction 3**

This reaction has been proposed to occur via formation of carbamoyl phosphate, 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 II). All four mutants showed no significant change in \( V_{\text{max}} \) when compared with the wild type enzyme. However, the

| Enzyme   | DNA sequence* |
|----------|---------------|
| K116Q    | 5'-CATGAGGATATTGCTGCCCATCAG-3' |
| K116A    | 5'-CATGAGGATATTGCTGCCCATCAG-3' |
| K159Q    | 5'-GGTCAGGATATTGCTGCCCATCAG-3' |
| E276Q    | 5'-CTGACAGAACAGGCAATTCTGCAGTCCCAG-3' |
| H209A    | 5'-CAAGTGGCCGAGCTTGGATATCAATCACCGGATGTCGAGTATCTC-3' |
| K116A    | 5'-CAAGTGGCCGAGCTTGGATATCAATCACCGGATGTCGAGTATCTC-3' |

* The underlined bases indicate the nucleotide positions that were changed.
TABLE II  
Kinetic parameters for the bicarbonate-dependent ATPase reaction  
The kinetic parameters were determined by varying [ATP] at a constant saturating level of bicarbonate or by varying [KHCO₃] while holding ATP at 25 mM and biotin at 60 mM. Standard errors on Kₘ and Vₘₐₓ were calculated from nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from Kₘ and Vₘₐₓ.

| Kₘ HCO₃ (mM) | Kₘ ATP (mM) | Vₘₐₓ (μM/min) | V/K ATP (μM/min) |
|--------------|-------------|----------------|------------------|
| Wild type    | 1.1 ± 0.3   | 0.08 ± 0.01    | 0.073 ± 0.001    | 0.9 ± 0.1       |
| H209A        | 16.7 ± 1.0  | 5.7 ± 1.2      | 0.043 ± 0.004    | 0.005 ± 0.002   |
| E276Q        | 1.9 ± 0.1   | 3.0 ± 0.2      | 0.072 ± 0.002    | 0.024 ± 0.002   |
| K159Q        | 1.8 ± 0.2   | 7.4 ± 1.4      | 0.087 ± 0.009    | 0.012 ± 0.004   |
| K116Q        | 1.3 ± 0.1   | 4.2 ± 0.8      | 0.075 ± 0.007    | 0.018 ± 0.005   |

* Data taken from Ref. 13.

TABLE III  
Kinetic parameters for the biotin-dependent ATPase reaction  
The kinetic parameters were determined by varying [biotin] at constant saturating levels of bicarbonate and 25 mM ATP. The S.E. on Vₘₐₓ and Kₘ were calculated from nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors from Kₘ and Vₘₐₓ.

| Kₘ biotin (mM) | Vₘₐₓ (μM/min) | V/K biotin (μM/min) |
|----------------|---------------|-------------------|
| Wild type      | 134 ± 12      | 78.6 ± 7          | 0.59 ± 0.1       |
| H209A          | 1234 ± 117    | 2.49 ± 0.17       | 0.0020 ± 0.0003  |
| E276Q          | 137 ± 24      | 0.90 ± 0.06       | 0.006 ± 0.001    |
| K159Q          | 125 ± 10      | 0.37 ± 0.01       | 0.0030 ± 0.0004  |
| K116Q          | _b            | _b                | _b               |

* Data taken from Ref. 13.  
* 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.

TABLE IV  
Ratio of ADP to carboxybiotin production  
The amounts of ADP and carboxybiotin formed over a 1-h period were quantitated as described under "Materials and Methods."

| Wild type      | 0.96 ± 0.18  |
|----------------|--------------|
| H209A          | 1.2 ± 0.1    |
| E276Q          | 0.86 ± 0.01  |
| K159Q          | 0.78 ± 0.16  |
| K116Q          | 1.1 ± 0.1    |

Since the ATPase assay measured 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 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 IV). All four mutants produced a ratio of carboxybiotin to ADP that was nearly 1:1. These results indicated 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.

\[
\text{MgADP + Carbamyl phosphate} \rightleftharpoons \text{MgATP + Carbamate}
\]

Reaction 4  
The carbamate rapidly decomposes to carbon dioxide and ammonia. Reaction 4 represents the reverse of Reaction 3, 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 V). The mutations did not have a significant effect on the Kₘ for either carbamyl phosphate or ADP. However, a modest decrease in Vₘₐₓ 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 concentration of ADP and carbamyl phosphate, both in the absence and presence of 60 mM biotin (Table VI). The degree of stimulation of the ATP synthesis activity by biotin was decreased 10-fold by the E276Q mutation, whereas the H209A mutant showed no significant decrease in stimulation. Both
K116Q and K159Q showed a decrease of ~2.5-fold compared with wild type (Table VI).

**Magnesium Assay**—Biotin carboxylase requires two equivalents of magnesium for activity. One equivalent is complexed to ATP, whereas 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 [MgCl2]. All four mutants exhibited a dependence on MgCl2 similar to that of wild type. This suggests that these mutations did not affect the affinity of the enzyme for magnesium (Fig. 1).

**DISCUSSION**

The 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 Km 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 Km value for ATP was consistent with the three-dimensional structure of biotin carboxylase bound to ATP as well as with the three-dimensional structures of other ATP-grasp enzymes bound to ADP or AMPPNP.1 The crystal structure of biotin carboxylase complexed with ATP revealed an electrostatic interaction between the β-amino group of Lys-116 and the α-phosphoryl oxygen of ATP (14). As shown in Table VII, the residues homologous to Lys-116 in enzymes of the ATP-grasp family also interacted with the oxygens of the α or β 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 Km for ATP (17).

Although all the available crystallographic and kinetic data implicate Lys-116 in binding an α or β 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. The degree of stimulation of the reaction by MgCl2 is expressed as a percentage of the maximum rate measured for each of the mutants and wild type.

### Table V

**Kinetic parameters for the ATP synthesis reaction**

The kinetic parameters were determined by varying carbamyl phosphate concentrations (CbmP) at constant saturating levels of ADP or varying [ADP] at constant saturating levels of CbmP. The S.E. on Km and Vmax were calculated from nonlinear regression analysis. The error on V/K was calculated by standard propagation of the errors on Km and Vmax.

|        | Km CbmP | Km ADP | Vmax | V/K ADP |
|--------|---------|--------|------|---------|
| Wild type | 4.8 ± 0.2 | 0.19 ± 0.01 | 0.31 ± 0.01 | 1.6 ± 0.1 |
| H209A | 7.3 ± 0.5 | 0.39 ± 0.07 | 0.12 ± 0.01 | 0.31 ± 0.08 |
| E276Q | 2.3 ± 0.1 | 0.42 ± 0.01 | 0.087 ± 0.001 | 0.21 ± 0.01 |
| K159Q | 3.0 ± 0.6 | 0.93 ± 0.08 | 0.12 ± 0.01 | 0.13 ± 0.02 |
| K116Q | 5.0 ± 0.7 | 0.31 ± 0.05 | 0.077 ± 0.003 | 0.25 ± 0.05 |

* Data taken from Ref. 26.

### Table VI

**Stimulation of ATP synthesis reaction**

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.

|        | Stimulation factor |
|--------|-------------------|
| Wild type | 25 |
| H209A | 21 |
| E276Q | 9 |
| K116Q | 10 |
| K159Q | 9 |

## FIG. 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 MgCl2. The degree of stimulation of the reaction by MgCl2 is expressed as a percentage of the maximum rate measured for each of the mutants and wild type.
These enzymes were chosen for structural comparison with biotin carboxylase because of their known three-dimensional structures with bound nucleotide or nucleotide analog.

| Residue in biotin carboxylase | n-Ala n-Ala ligase<sup>a</sup> | Carbamyl phosphate synthetase, carboxyphosphate domain<sup>b</sup> | Carbamyl phosphate synthetase, carboxyphosphate domain<sup>b</sup> | N<sup>6</sup>-Carboxyaminomimidazole ribonucleotide synthetase<sup>c</sup> | Glycinamide ribonucleotide transformylase<sup>d</sup> |
|-------------------------------|-------------------------------|-----------------------------|-----------------------------|-------------------------------|-----------------------------|
| Lys-116                       | Lys-97                        | Arg-129 Hydrogen bonds with α and β phosphoryl groups of ATP | Arg-675 Hydrogen bonds with α and β phosphoryl groups of ATP | Arg-80 Hydrogen bonds with β phosphoryl group of ATP | Arg-114 Hydrogen bonds with β phosphoryl group of ATP |
| Lys-159                       | Lys-144 Hydrogen bonds to ribose hydroxyl groups coordinated to magnesium | Arg-169 Hydrogen bonds to ribose hydroxyl groups | Arg-715 Hydrogen bonds to ribose hydroxyl groups | Lys-120 Hydrogen bonds to ribose hydroxyl groups and N7 of adenine | Lys-155 Hydrogen bonds to ribose hydroxyl groups |
| His-209                       | Glu-187 Coordinated to magnesium | Glu-215 Hydrogen bonds to ribose hydroxyl groups | Glu-761 Hydrogen bonds to ribose hydroxyl groups | Glu-161 Hydrogen bonds to ribose hydroxyl groups | Glu-203 Hydrogen bonds to ribose hydroxyl groups |
| Glu-276                       | Asp-257 Coordinated to magnesium | Hydrogen bonds to ribose hydroxyl groups coordinated to magnesium | Glu-257 Hydrogen bonds to ribose hydroxyl groups coordinated to magnesium | Glu-267 Hydrogen bonds to ribose hydroxyl groups coordinated to magnesium | Glu-267 Hydrogen bonds to ribose hydroxyl groups coordinated to magnesium |

<sup>a–d</sup> Data taken from Refs. 6, 27, 11, and 12, respectively.
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 $V_{\text{max}}$ 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 the binding of biotin to the enzyme position 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 to calculate the binding energy, a relatively low value of 1.2 kcal/mol 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 a 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.

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