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**Evidence that Cys230 and Lys238 of  
Biotin Carboxylase do not act as an Acid-  
Base Pair to Deprotonate Biotin**

**Brett Lloyd**

**Submitted: April 28, 1999**

***An Honor's Thesis: In partial fulfillment of College Honor's for  
the Bachelor's of Science Degree in Biochemistry.***

**Louisiana State University  
Baton Rouge, Louisiana**

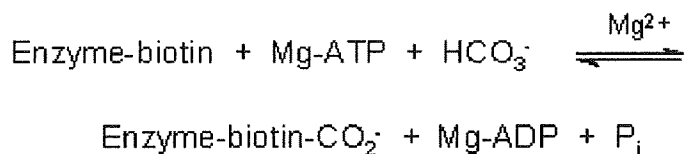
## **Abbreviations**

- 1) ATP - adenosine triphosphate
- 2) NEM - *N*-ethylmaleimide
- 3) o-PA - o-phthalaldehyde
- 4) DNA - deoxyribonucleic acid
- 5) PCR - polymerase chain reaction
- 6) EDTA - ethylenediaminetetraacetic acid
- 7) ADP - adenosine diphosphate
- 8) NADH - nicotinamide adenosine triphosphate
- 9) HEPES - [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]
- 10) NADP - nicotinamide adenine dinucleotide phosphate
- 11) PEP - phosphoenolpyruvate
- 12) WTBC - wild-type biotin carboxylase

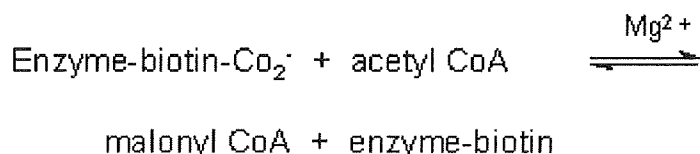
**Abstract** Acetyl-CoA carboxylase is responsible for the conversion of acetyl-CoA to malonyl CoA in long chain fatty acid synthesis. In *Escherichia coli*, this enzyme is comprised of biotin carboxylase, carboxytransferase, and biotin carboxyl carrier protein. Using a system described by Blanchard et. al., site-directed mutagenesis was performed to mutate Lys238 to glutamine and Cys230 to alanine of biotin carboxylase in an attempt to characterize residues. Under this system, these mutants of biotin carboxylase were purified free of contamination from the wild-type enzyme derived from the chromosomal copy of the gene. It has been proposed that these residues act as an acid-base pair in the active site of the biotin carboxylase and are responsible for the deprotonation of the N-1 nitrogen of biotin. The K238Q and C230A mutants display  $K_m$  values for ATP that are at least 50-fold larger than wild-type. These results suggest that Lys238 and Cys230 are important in binding ATP. Also, the ATPase reaction was not stimulated by the addition of biotin for K238Q. This also infers that K238Q might play a role in the extraction of the proton from biotin. The C230A mutation, on the other hand, did not have a significant effect on the carboxylation reaction indicating that it is not catalytically involved in the deprotonation of biotin. The results show that this hypothesis does not correctly describe the deprotonation of biotin.

## Introduction

Acetyl CoA carboxylase in *E. coli* is responsible for catalyzing the first dependent and rate-determining step of long chain fatty acid synthesis. The overall reaction involves the conversion of acetyl-CoA to malonyl CoA in two separate half reactions (reactions 1 and 2). These reactions are carried out by three subunits in the bacterial form of the enzyme. The subunits are biotin carboxylase, carboxyltransferase, and the biotin carboxyl carrier protein. Biotin carboxylase catalyzes the first-half reaction, involving the ATP-dependent carboxylation of biotin using bicarbonate as the CO<sub>2</sub> source. Carboxyltransferase catalyzes the second half-reaction, in which the carboxyl group is transferred from biotin to acetyl CoA to form malonyl CoA. *In vivo*, biotin is attached to the biotin carboxyl carrier protein (denoted enzyme-biotin in reaction 1) via an amide linkage to a lysine residue. In animals, the three subunits of acetyl CoA carboxylase are on the same peptide chain, making it difficult to study the function of individual subunits. In bacteria, each subunit is a separate gene product. Upon isolation, biotin carboxylase and carboxyltransferase retain activity, and both utilize free d-biotin as a substrate (11). Additionally, both biotin carboxylase and carboxyltransferase have been overexpressed, allowing ample amounts of protein for kinetic studies. Thus, biotin carboxylase and carboxyltransferase, derived from *E. coli*, provide an excellent model system for mechanistic studies of biotin-dependent carboxylases.



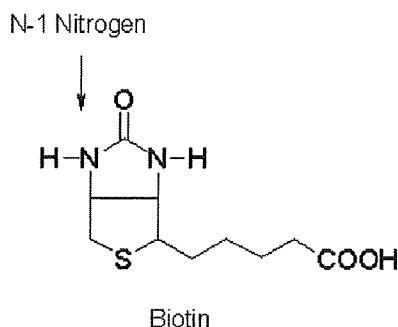
**Reaction 1.**



### Reaction 2.

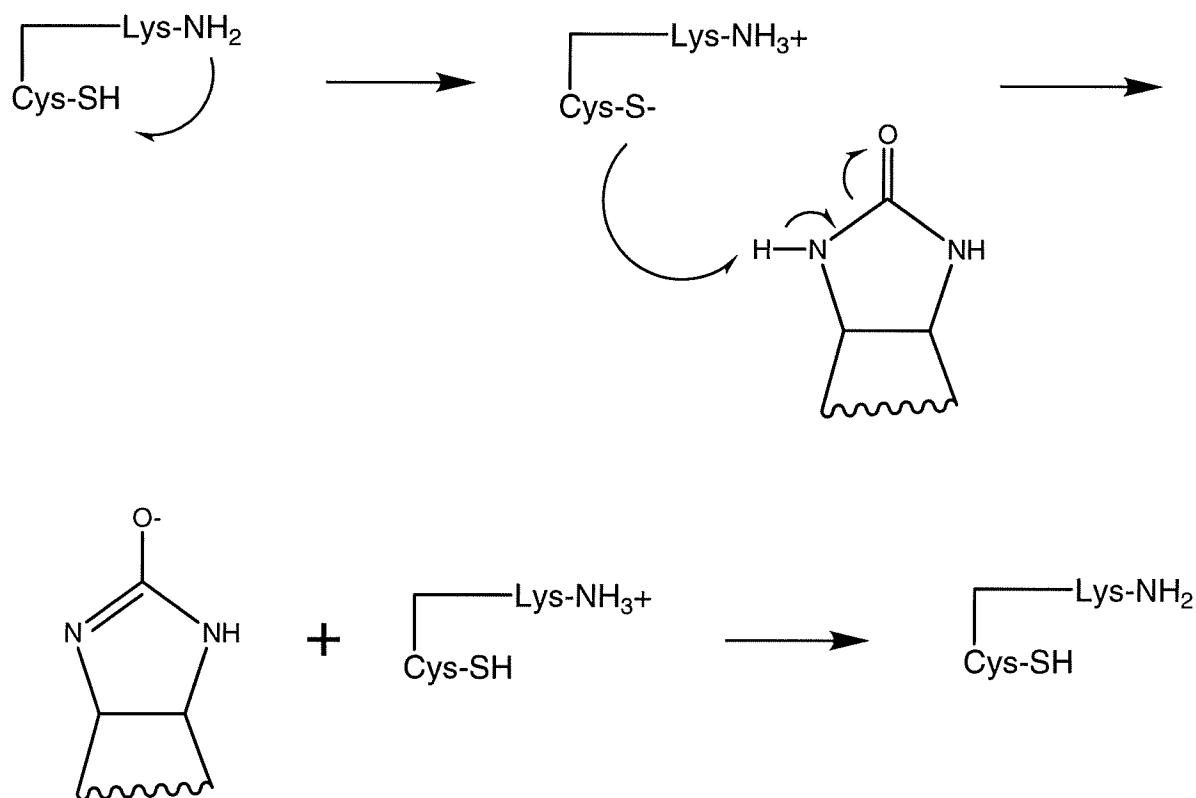
The first half-reaction catalyzed by biotin carboxylase requires ATP to bind to the enzyme followed by bicarbonate in a stepwise mechanism. This reaction produces a carboxyphosphate intermediate. The carboxyphosphate intermediate formed is very unstable and has an estimated half-life of 70 msec (12). Next, the N-1 nitrogen of biotin attacks carboxyphosphate. This forms carboxybiotin and inorganic phosphate (11). A partial forward reaction, the ATPase reaction, may occur in the absence of biotin. In this reaction, biotin carboxylase catalyzes the ATP-dependent conversion of bicarbonate to carboxyphosphate and ADP. In comparison to the overall reaction or biotin-dependent ATPase reaction (reaction 1), the rate of the ATPase reaction is very slow. The addition of biotin to the ATPase reaction stimulates the rate 1100-fold (5).

When examining the mechanism of biotin carboxylase, it is apparent that a primary problem is the N-1 nitrogen of biotin is not very nucleophilic. Therefore, biotin is unable to attack the carboxyphosphate intermediate. In order for the N-1 nitrogen of biotin to become nucleophilic, the proton must be extracted from that position (Figure 1). It has been proposed that a lysine-cysteine pair in close proximity may be responsible for the extraction of this proton (9).



**Figure 1. Free d-biotin.**

First, the lysine extracts the proton from a nearby cysteine residue (Figure 2). Next, the thiolate anion acts as a catalytic base and extracts the proton from the N-1 nitrogen of biotin, thus activating biotin in the enol form. At physiological pH, the lysine residue becomes deprotonated and the enzyme returns to its initial state. This mechanism was originally based on characterization studies of biotin carboxylase by Tipton and Cleland (13). In this study, biotin carboxylase was found to be susceptible to inactivation by the sulfhydryl-modifying agent (Figure 3) *N*-ethylmaleimide (NEM). However, in the presence of substrates biotin carboxylase was protected against inactivation by NEM. Further support of a cysteine-lysine pair being involved in the deprotonation of biotin was offered by Wernberg and Ash's (15) inactivation studies of pyruvate carboxylase by *o*-phthalaldehyde (*o*-PA). The reagent *o*-PA has been shown to cross-link lysine and cysteine residues that are separated by approximately 3 Å. In their study, pyruvate carboxylase was shown to be inactivated by *o*-PA, suggesting the presence of an essential cysteine-lysine pair. Additionally, the crystal structure of biotin carboxylase has been solved by x-ray crystallography (14) and shows the active site cysteine-lysine pair believed to be involved in catalysis to be 4.2 Å apart, a sufficient distance for the mechanism to occur.



**Figure 2. Proposed mechanism for Lys238 and Cys230.**



In this report, we further test the hypothesis that a cysteine-lysine pair is involved in the deprotonation of biotin in biotin carboxylase catalysis. To do this, Lys238 was mutated to glutamine in biotin carboxylase such that the amide side chain cannot act as a catalytic base. Furthermore, Cys230 in biotin carboxylase was mutated to an alanine residue to eliminate the thiol proton. The results indicate that this hypothesis is incorrect and that Cys230 and Lys238 play a role in binding ATP, while Lys238 interacts with biotin.

## **Materials and Methods**

Chemicals and Enzymes. *Escherichia coli* strain JM109, plasmid pGEM-7zf, and the Wizard plasmid purification and isolation kit were purchased from Promega. The expression vectors, pET14b and pET28b, *E. coli* strain BL21(DE3)pLysS, and His•binding resin were from Novagen. Restriction enzymes, Deep vent polymerase, and T4 DNA ligase were from New England Biolabs. The primers were synthesized by Life Technologies GibcoBRL.  $^{14}\text{C}$  labeled sodium bicarbonate with an activity of 0.1 mCi/mmol was from Amersham. Pyruvate kinase was from Boehringer Mannheim. All other reagents and chemicals were from Sigma or Aldrich.

Construction of a Histidine•tagged Biotin Carboxylase. A six-histidine tag was added to the N-terminus of the plasmid copy of biotin carboxylase. This enabled the separation of the chromosomal copy of biotin carboxylase from the plasmid copy. The template copy of the biotin carboxylase gene was a gift from Dr. John Cronan of the University of Illinois. The addition of the poly-histidine tag and the introduction of restriction sites EcoRI and NdeI on the 5' of the gene and BamHI site onto the 3' end of the biotin carboxylase gene was accomplished using the polymerase chain reaction (PCR). The

PCR product and pGEM-7f were cut with EcoRI and BamHI and ligated to form the plasmid, pGLW1. The biotin carboxylase gene was then sequenced to verify that no mutations had been introduced. Next, pGLW1 and pET14b, the expression plasmid, were cut with NdeI and BamHI and ligated to form pGLW2. The 5' end of pGLW2 was extended by 20 amino acids including 6 contiguous histidine residues that would be transcribed on the N-terminus of the protein.

Site-Directed Mutagenesis. The PCR overlap extension method (8) was used to introduce mutations into the biotin carboxylase gene. The template was pGLW1 and the flanking primers were those used in construction of pGLW1. The pair of internal oligonucleotides used to construct the K238Q mutant are:

(1) 3' TCTTCGACCACTTGCTGGTGGCGGC 5'

(2) 5' GCCGCCACCAGCAAGTGGTCGAAGA 3'

The pair of internal oligonucleotides used to construct the C230A mutant are:

(3) 3' CGCCTTGCACTGCGGAGGTACGTTGCG 5'

(4) 5' GCGGAACGTGACGCCTCCATGCAACGC 3'

The PCR product and pGEM-7f were cut by EcoRI and BamHI and ligated to form pRBL3, containing the K238Q mutation, and pRBL4, containing the C230A mutation. Next, both pRBL3 and pRBL4 and pET28b, the expression plasmid, were cut by NdeI and BamHI and ligated to form pRBL5 and pRBL6, respectively. The genes for the biotin carboxylase mutants were sequenced to ensure that the proper mutations had been made and that no other mutations had been introduced into the sequence. The verifications of the mutations are presented in figures 5 and 6 for K238Q and C230A,

respectively. The upper image shows the sequence of the mutant, while the lower image is the sequence for wild-type biotin carboxylase.

Growth and Purification of the Wild-Type and Mutant Biotin Carboxylases. Both pRBL5 and pRBL6 were transformed into *E. coli* strain BL21(DE3)pLysS. The cultures were started from an overnight consisting of 10 mL of LB media, 100 µg/ mL ampicillin for the wild-type or 30 µg/ mL of kanamycin for the mutants. The cultures were grown in 2 L flasks at 37 °C containing 1 L of LB media. Cultures were grown to midlog phase (approximately 2 hours) and induced with 1 gram of lactose. The cultures were incubated for another 2 hours and centrifuged at 10,400 x g, 4 °C for 10 minutes.

The cell pellets were resuspended in 40 mL of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) for each 1 L growth. Cells were lysed by the freeze-thaw method in which the cells were frozen and thawed three times. DNase was added to degrade the DNA in the lysate. The lysed cell solution was centrifuged for an hour at 20,200 x g, 4° C. The supernatant was filtered using a 0.4 µm filter and 0.1% of Triton X-100 was added to prevent non-specific binding to the His-binding resin.

Wild type and biotin carboxylase mutants were purified using His-binding resin. Purification columns were poured with 2.5 mL of His•Tag resin and washed with 7.5 mL of ddH<sub>2</sub>O. The columns were charged with 12.5 mL of charge buffer (50 mM NiSO<sub>4</sub>) and equilibrated with 7.5 mL of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). The protein was applied to the columns and followed by 25 mL of binding buffer to remove proteins without the His•Tag. The columns were washed with 15 mL of a 60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9 solution and the protein was eluted with a 500 mM imidazole, 250 mM NaCl, 10 mM Tris-HCl pH 7.9

solution. The columns were stripped and stored using a 50 mM EDTA, 250 mM NaCl, 10 mM Tris-HCl pH 7.9 solution. The protein solution was dialyzed overnight in 2 L of a 0.67 mM EDTA, 10 mM KHPO<sub>4</sub>, pH 7.0 buffer. The protein was precipitated with ammonium sulfate at 60% saturation for 10 minutes and centrifuged at 13,000 x g for 10 minutes at 4 °C. The pellet was resuspended in 500 mM KCl, 10 mM Hepes, pH 7.0 and dialyzed against this buffer overnight. Biotin carboxylase was concentrated by vacuum dialysis using a colloidion bag and the concentration was determined using the method of Bradford (4).

Removal of the His-Tag Since the histidine tag will bind divalent cations, the His-tag was removed from biotin carboxylase for all experiments in which Mg<sup>2+</sup> was varied. Initially, the protein was dialyzed overnight against 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, and 120 mM Tris-HCl, pH 8.4. Restriction grade thrombin was added to the biotin carboxylase solution at a ratio of 1 unit thrombin to 1 mg of biotin carboxylase and incubated for 16 hours at room temperature. The protein was loaded on the his-binding columns and the columns were washed with binding buffer to remove any thrombin. Cleaved biotin carboxylase was eluted with 60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9. Once the cleaved protein was collected, it was dialyzed and concentrated as described earlier. The uncut protein that remained bound to the histidine column was also eluted later with the standard elute buffer.

Bicarbonate-Dependent ATPase/ Biotin-Dependent ATPase Reactions. Unless stated otherwise, 0.5 mL of the reaction mixtures were monitored at 340 nm in 0.5 mL quartz cuvettes with a path length of 1 cm using a Uvikon 810 (Kontron Instruments) spectrophotometer, connected to a PC with a data acquisition program. The temperature

was maintained at 25 °C by a circulating water bath to keep the cuvette temperature constant. The kinetic parameters for ATP hydrolysis by biotin carboxylase and mutants were studied in the presence and absence of biotin. The production of ADP was coupled to pyruvate kinase and lactate dehydrogenase and the disappearance of NADH was monitored spectrophotometrically for the ATPase and biotin reactions (reaction 3 and 4). The assay mixture contained 18 units of lactate dehydrogenase, 10 units of pyruvate kinase, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM MgCl<sub>2</sub>, and 100 mM HEPES at pH 8.0. The reaction was started with the addition of enzyme to the assay solution. For the ATPase assay (reaction 3), the concentration of ATP was varied while holding the concentration of bicarbonate constant at ten times the  $K_m$ . During the biotin assay (Reaction 4), ATP and bicarbonate were held constant at saturating levels and the concentration of biotin was varied. The biotin used in this assay had one mole of potassium per mole of biotin. Since the  $K_m$  for biotin is high, the ionic strength of the assay solution was held constant by addition of KCl.

To obtain the  $K_m$  for bicarbonate, special precautions had to be taken since the endogenous level of bicarbonate at pH 8.0 is 0.5 mM (2), which is close to the  $K_m$  for bicarbonate for wild-type biotin carboxylase at  $0.37 \pm 0.04$  mM. Endogenous bicarbonate was removed using a procedure described by Blanchard et. al. (3), such that bicarbonate could be varied below its  $K_m$  value. To remove the endogenous bicarbonate deionized water was boiled for 10 minutes and cooled in a glove bag under a nitrogen atmosphere. A stock solution of HEPES at pH 8.0 was prepared under nitrogen in the glove bag. Stock solutions of ATP, NADH, pyruvate kinase, phosphoenolpyruvate, and lactate dehydrogenase were made in glass vials, capped with septa, and flushed with

nitrogen. All nitrogen was passed through an Ascarite column to remove any CO<sub>2</sub>. HEPES was added to the vials using a Hamilton syringe. A cocktail was made containing all assay components except bicarbonate and enzyme. Each assay was run in a volume of 1 mL containing 3 mM ATP, 60 mM biotin, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM MgCl<sub>2</sub>, 20 units of pyruvate kinase, 36 units of lactate dehydrogenase, and 100 mM HEPES. A stock bicarbonate solution was prepared and stored in a gas-tight flask. All reactions were performed in 1 mL quartz cuvettes with a 1 cm path length, covered with a rubber septum. The cuvettes were initially purged with nitrogen, the assay stock was added with a Hamilton syringe, and the reaction was started with the addition of enzyme.

Carboxybiotin Assay. The amount of carboxybiotin was determined using a modification of the [<sup>14</sup>C]bicarbonate fixation method designed by Guchhait et. al. (7). The reactions were run in 1.5 mL microfuge tubes for an hour at 25 °C. The reaction mixture (0.5 mL) contained 9 mM ATP, 8 mM MgCl<sub>2</sub>, 100 mM biotin, 70 mM KHCO<sub>3</sub>, and 100 mM HEPES, pH 8.0, supplemented with 3.5 µL (17.5 nCi) of NaH<sup>14</sup>CO<sub>3</sub>. The reaction was started by the addition of enzyme, allowed to react for one hour, and stopped by the addition of 3.6 mL of saturated KOH. The unreacted carbonate was precipitated with 200 µL of 2.0 M BaCl<sub>2</sub>/ 0.1 M Ba(OH)<sub>2</sub>. After incubating for 2 minutes, the tubes were centrifuged at 1000 x g for 5 minutes. A 100 µL sample was removed from the supernatant and counted in a scintillation vial to measure the <sup>14</sup>C activity. Also, an aliquot of the reaction mixture with no enzyme and without precipitation by BaCl<sub>2</sub>/ Ba(OH)<sub>2</sub> was counted to measure the specific activity of bicarbonate. From this specific activity the amount of carboxybiotin was calculated.

Next, the amount of ADP produced was measured to determine the ratio of carboxybiotin to ADP produced (reaction 1). The reaction conditions were the same as the carboxybiotin assay except no radiolabeled bicarbonate was added. Immediately after mixing all components, an aliquot was removed and the amount of ATP was measured by end-point analysis. End-point analysis was performed in a total volume of 1.0 mL containing 7.5 units of hexokinase, 5 units of glucose-6-phosphate dehydrogenase, 0.5 mM glucose, 0.4 mM NADP, 8 mM MgCl<sub>2</sub>, and 100 mM HEPES at pH 8.0. After one hour, a second aliquot was removed and the amount of ADP produced was determined from the difference between the amount of ATP present at the beginning of the reaction and the amount of ATP remaining after 1 hour incubation.

NEM Modification Assay. Enzyme modification by NEM was carried out in 10 mM HEPES, 500 mM KCl, pH 7.0. The modification reaction was initiated by the addition of 6.1 mM NEM to the biotin carboxylase solution. At various time intervals, aliquots were removed and assayed for activity. To determine activity, the same conditions used for the biotin-dependent ATPase assay were utilized (reaction 4) except that ATP was held constant at 3 mM. The activity of biotin carboxylase without any NEM added was measured and used to calculate the percent activity remaining after incubation with NEM.

Data Analysis. The  $V_{max}$  and  $K_m$  were obtained by fitting the data to the Michaelis-Menten equation (equation 1) using the program Enzfitter:

$$v = V_{max} * [S] / (K_m + [S]) \quad (1)$$

In equation 1,  $v$  is the initial velocity,  $V_{max}$  is the maximal velocity,  $[S]$  is the concentration of substrate, and  $K_m$  is the Michaelis constant.

## Results

Separation of the Mutant Biotin Carboxylase from the Chromosomal Copy. A histidine-tag was added to biotin carboxylase mutants to separate them from the wild type protein produced from the chromosomal copy of the biotin carboxylase gene. During cell growth, both the chromosomal and plasmid copies of the biotin carboxylase gene are transcribed, so this step allows for the removal of the chromosomal copy of the protein. The engineered protein was then separated from the chromosomal protein using histidine columns. Any protein not containing the histidine-tag will be eluted under lower concentrations of imidazole, while high concentrations are required to elute the enzymes containing the His-tag. When biotin carboxylase was assayed with a His-tag and without the His-tag, the same results were obtained. Therefore, the His-tag did not have an effect on the activity of biotin carboxylase.

The Bicarbonate-Dependent ATPase Reaction. Biotin carboxylase will catalyze the hydrolysis of ATP in the absence of biotin, but at a much slower rate. The  $K_m$  for ATP and the  $V_{max}$  of the ATP hydrolysis reaction were determined for the wild-type enzyme, K238Q, and C230A and are shown in Table 1. The  $V_{max}$  values were  $0.12 \pm 0.01 \text{ min}^{-1}$  and  $0.10 \pm 0.01 \text{ min}^{-1}$  for K238Q and C230A, respectively. These values were not significantly different from the wild-type value of  $0.07 \pm 0.01 \text{ min}^{-1}$ . The  $K_m$  for ATP for WTBC, K238Q, and C230A were  $0.08 \pm 0.01 \text{ mM}$ ,  $6.87 \pm 0.27 \text{ mM}$ , and  $4.23 \pm 0.13 \text{ mM}$ , respectively. K238Q displayed an 86-fold increase in the  $K_m$  for ATP, while C230A exhibited a 53-fold increase in the  $K_m$  for ATP. Hence, the  $V/K$  values were significantly lower for the mutants than wild-type. The  $V/K$  for wild-type was  $0.90 \pm 0.05 \text{ min}^{-1} \text{ mM}^{-1}$  while K238Q and C230A both displayed a  $V/K$  of  $0.02 \pm 0.01 \text{ min}^{-1} \text{ mM}^{-1}$ .



<sup>1</sup>, respectively. These results demonstrate a significant decrease in the catalytic efficiency of the ATPase assay for both K238Q and C230A.

| <b>Table 1: Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction</b> |                                  |                 |  |
|--|----------------------------------|-----------------|--|
|  | $V_{\max}$ ( $\text{min}^{-1}$ ) | $K_m$ (mM)      | $V/K$ ( $\text{min}^{-1} \text{mM}^{-1}$ ) |
| WTBC   | $0.07 \pm 0.01$                  | $0.08 \pm 0.01$ | $0.90 \pm 0.05$                            |
| K238Q  | $0.12 \pm 0.01$                  | $6.87 \pm 0.27$ | $0.02 \pm 0.01$                            |
| C230A  | $0.10 \pm 0.01$                  | $4.23 \pm 0.13$ | $0.02 \pm 0.01$                            |

Since the wild-type  $K_m$  for bicarbonate at  $0.37 \pm 0.04$  mM (3) is nearly the same as the endogenous level of bicarbonate, then all solutions had to be degassed to remove bicarbonate. Once the endogenous bicarbonate was removed from all solutions, then bicarbonate was varied to determine the apparent  $K_m$ . The  $K_m$  for bicarbonate could not be determined for the K238Q mutant. It is possible that the mutation decreased the  $K_m$  of bicarbonate for K238Q to a level equivalent to the residual level of bicarbonate. The apparent  $K_m$  for the C230A mutant was  $0.66 \pm 0.07$  mM, which was not substantially different than wild-type.

The Biotin-Dependent ATPase Reaction. When biotin is added, the ATPase reaction is stimulated and carboxybiotin is produced (reaction 4). Under these conditions, biotin was varied while ATP and bicarbonate were held at constant, saturating levels. The data for these parameters are displayed in Table 2. The  $K_m$  for biotin for wild-type and C230A were similar at  $134.0 \pm 13.8$  mM and  $167.5 \pm 17.9$  mM, respectively. The  $V_{\max}$  for C230A was slightly decreased to  $30.8 \pm 1.6 \text{ min}^{-1}$  from the wild-type value of  $78.6 \pm 3.2 \text{ min}^{-1}$ . The C230A mutation did not result in a significant change indicating that the binding of biotin to the enzyme was not affected and that Cys230 does not interact with biotin. In contrast, biotin did not stimulate the ATPase activity of the K238Q mutant.

| <b>Table 2: Kinetic Parameters for the Biotin-Dependent ATPase Reaction</b> |                                  |                  |  |
|---|----------------------------------|------------------|--|
|   | $V_{\max}$ ( $\text{min}^{-1}$ ) | $K_m$ (mM)       | $V/K$ ( $\text{min}^{-1} \text{mM}^{-1}$ ) |
| WTBC  | $78.6 \pm 3.2$                   | $134.0 \pm 13.8$ | $0.59 \pm 0.08$                            |
| K238Q   | No effect                        | No effect        | No effect                                  |
| C230A   | $30.8 \pm 1.6$                   | $167.5 \pm 17.9$ | $0.18 \pm 0.13$                            |

Since the ATPase assay involved detecting the production of ATP with the coupling enzymes pyruvate kinase and lactate dehydrogenase, there was a question as to whether the mutant enzymes were producing carboxybiotin. For example, has the hydrolysis of ATP been uncoupled from the formation of carboxybiotin, suggesting that the mutations have affected the carboxyl transfer step? Or, do the mutant enzymes show a 1:1 stoichiometry for the formation of carboxybiotin? To determine the answer to these questions, the carboxybiotin assay was performed. Ratios were determined for the amount of ADP produced to the amount of carboxybiotin produced for wild-type. The wild-type gave a 1:1 ratio according to the stoichiometry of the reaction, and C230A gave a 1:0.94 ratio of  $[\text{ADP}]:[^{14}\text{CO}_2\text{-biotin}]$ . The amount of carboxybiotin produced by K238Q was not detected by this assay, even though K238Q was able to hydrolyze ATP at the same rate as wild-type in the ATPase reaction (reaction 3). Carboxybiotin may not have been detected for the carboxybiotin assay, since the assay was not sensitive enough for measuring small amounts of carboxybiotin, or K238Q may not be making carboxybiotin.

Effects of Magnesium. Two equivalents of magnesium are required for biotin carboxylase activity. One equivalent of magnesium is bound to ATP and the function of the other equivalent is unknown. The initial velocity was measured at increasing concentrations of magnesium. The C230A mutant required a higher concentration of magnesium to reach 100% activity (figure 7), since its curve was pushed to the right side

of the graph. This is consistent with the rise in  $K_m$  for ATP. On the other hand, K238Q required less magnesium to reach 100% activity suggesting magnesium may be binding tighter to K238Q.

To determine if the C230A mutation may have increased the  $K_m$  for magnesium not bound to ATP, the enzyme was assayed by varying the level of free magnesium. The  $Mg^{2+}$  ATP complex was held constant at 6 mM. The apparent  $K_m$  for magnesium for wild-type and C230A were determined to be  $0.28 \pm 0.03$  mM and  $0.29 \pm 0.03$  mM, respectively. This indicated that Cys230 was not directly binding magnesium.

NEM Modification. The compound *N*-ethylmaleimide (NEM) inactivates wild-type biotin carboxylase (13). To determine if Cys230 and Lys238 were reacting with NEM, the incubation of NEM with C230A and K238Q by NEM were examined (Figure 8). Both wild-type and C230A were inactivated to 25% of the original activity after 25 minutes. The rate of inactivation by NEM for wild-type and C230A was 0.0632 and 0.0448, respectively. The K238Q mutant, on the other hand, did not inactivate under these conditions. After 25 minutes had passed, the K238Q mutant still retained 90% of its activity, indicating that NEM was actually inactivating Lys238 in the wild-type enzyme.

## **Discussion**

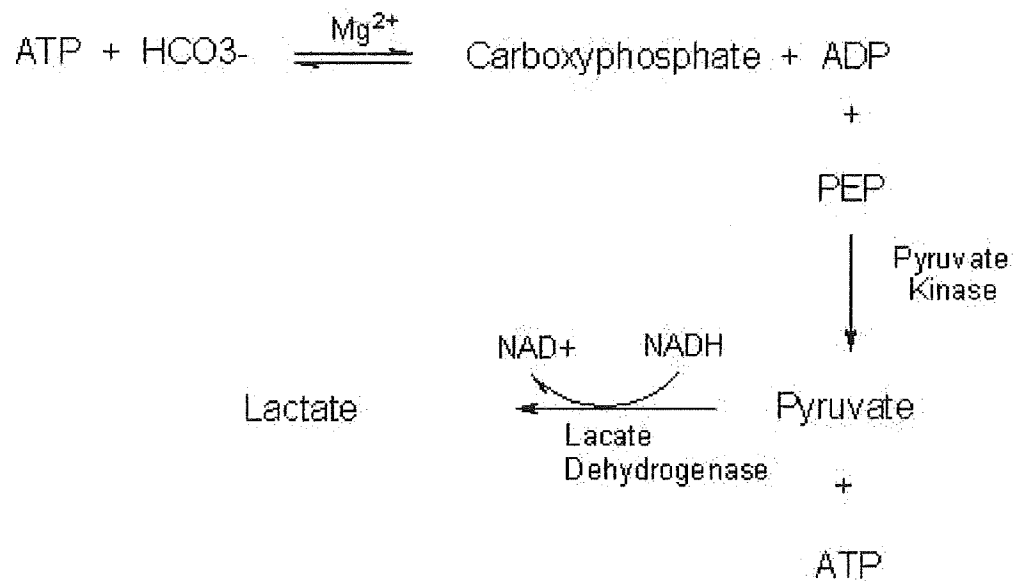
In the bicarbonate-dependent ATPase reaction (reaction 3), the maximal velocity was unchanged by either mutation, however, the  $K_m$  for ATP for the mutants increased at least fifty-fold (Table 1). The rise in  $K_m$  for ATP suggests that C230A and K238Q are both involved in binding ATP, but are not catalytically involved in the ATPase reaction.

When biotin is added, the rate of the ATPase reaction is stimulated 1100-fold in the wild-type enzyme (3). The C230A mutant has a  $K_m$  for biotin that is the same as wild-type and a slightly decreased  $V_{max}$ , suggesting that Cys230 is not required for catalysis in the biotin-dependent ATPase reaction. The K238Q mutant, on the other hand, showed a unique result since there was no increase in the velocity of the biotin-dependent ATPase reaction upon addition of biotin. This data suggests two possibilities. Either there is a drastic increase in the  $K_m$  for biotin, so K238Q no longer has a strong affinity for biotin, or K238Q is catalytically involved in the carboxylation of biotin. If K238Q is involved in the carboxylation of biotin, then the ability of K238Q to synthesize carboxybiotin would be reduced or eliminated.

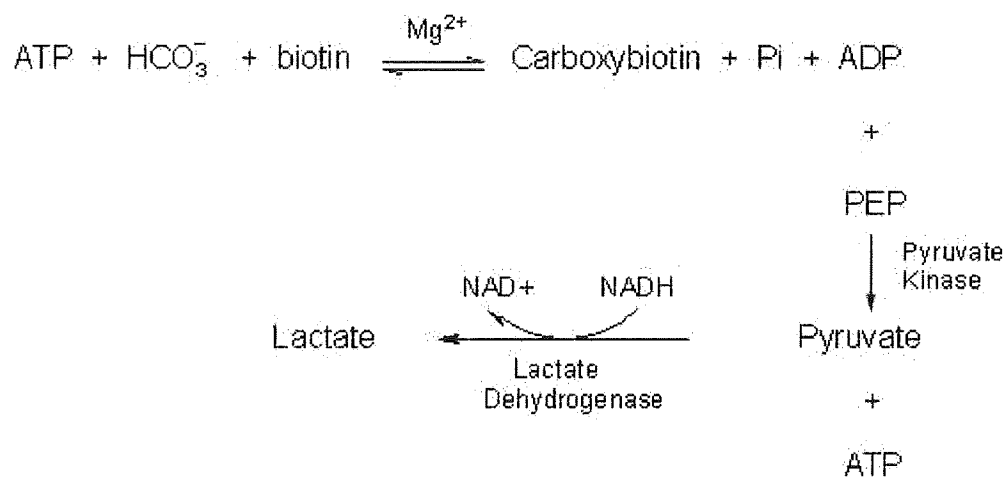
The stoichiometry of the first half-reaction (reaction 1) dictates that for each molecule of ATP, biotin, and bicarbonate consumed one molecule of ADP, carboxybiotin, and inorganic phosphate will be produced. It was confirmed that the ratio of carboxybiotin to ADP produced by the wild-type enzyme is 1:1. In addition, the C230A ratio was approximately this value. This signifies that C230A is still able to hydrolyze ATP and synthesize carboxybiotin. This evidence further supports that C230A is not involved in the carboxylation of biotin. The assay was unable to detect the production of carboxybiotin by the K238Q mutant. One possibility for this result is the assay is not very sensitive in detecting low levels of carboxybiotin. Since the  $V_{max}$  for the biotin-dependent ATPase reaction catalyzed by biotin is significantly lower than both wild-type and C230A, it is possible that only low levels of carboxybiotin are being produced by K238Q. Alternatively, it is also possible that K238Q is no longer able to make carboxybiotin.

Studies by Tipton and Cleland (13) show that wild-type biotin carboxylase is inactivated by NEM, while incubation of the enzyme with substrates before addition of NEM will protect the enzyme against modification. Since the rate of inactivation of C230A was the same as wild-type, NEM is not inactivating this particular cysteine residue. On the other hand, the reaction rate for K238Q was unaffected by incubation with NEM. This suggests that NEM is actually reacting with lysine (figure 4), specifically Lys238. Therefore, the NEM data does not support the hypothesis that a cysteine residue is involved in the deprotonation of the N-1 nitrogen of biotin.

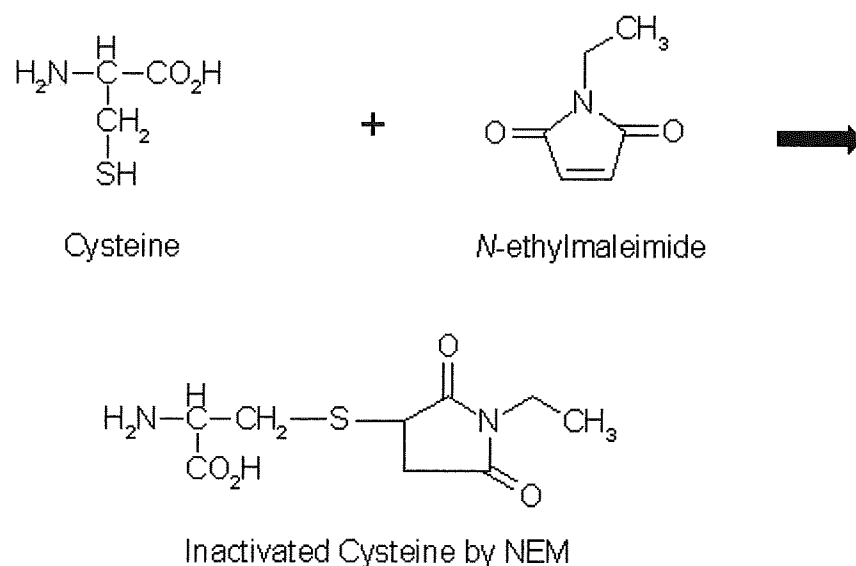
Overall, it may be concluded that the mechanism proposed by Wernberg and Ash for the deprotonation of biotin by a cysteine-lysine acid-base pair is not accurate. Since the  $K_m$  for biotin,  $K_m$  for magnesium, and the  $V_{max}$  for the biotin-dependent ATPase reaction for C230A are the same as wild-type and the ratio of carboxybiotin to ADP being produced is 1:1, C230A is not involved in the deprotonation or the carboxylation of biotin. However, because the  $K_m$  for ATP is increased, Cys230 is indicated in binding ATP. The K238Q mutant also has a  $K_m$  for ATP that is 86-fold larger than wild-type. This residue also appears to play a direct role in binding ATP. Since biotin no longer stimulates K238Q, it is possible that Lys238 also plays an important part in the deprotonation of the N-1 proton from biotin or the carboxylation of biotin.



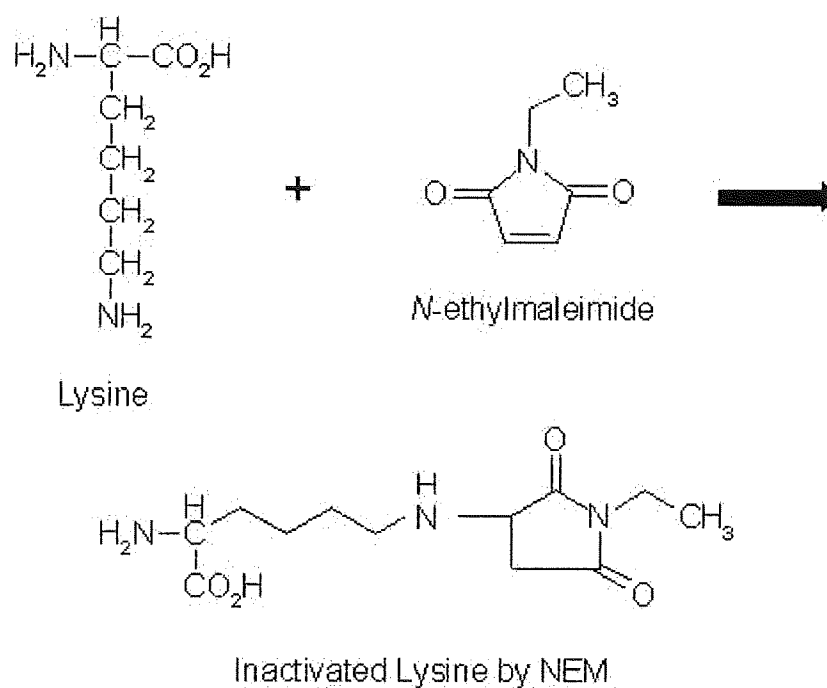
**Reaction 3. The Coupled Bicarbonate-Dependent ATPase Reaction.**



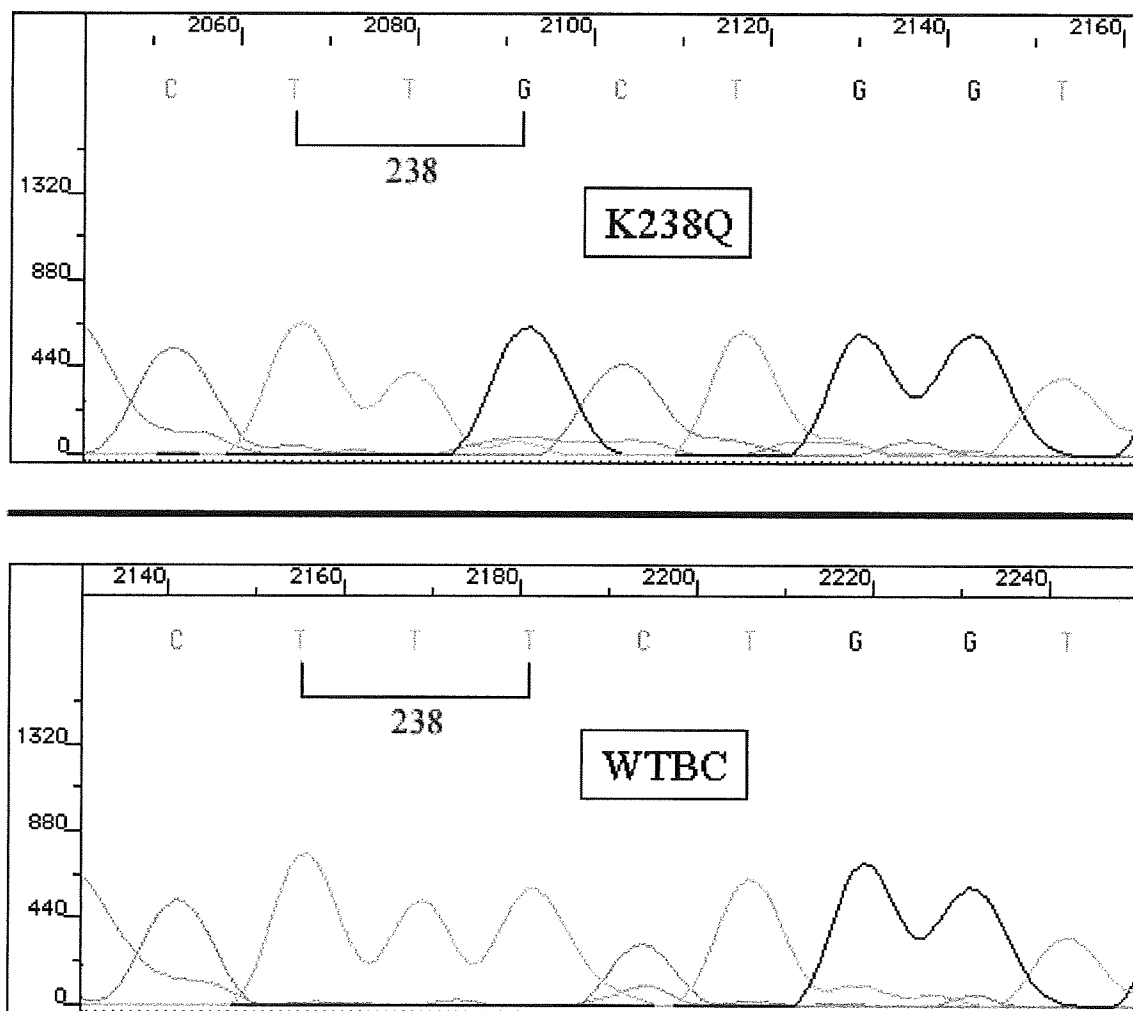
**Reaction 4. The Coupled Biotin-Dependent ATPase Reaction.**



**Figure 3. Inactivation of cysteine by NEM.**

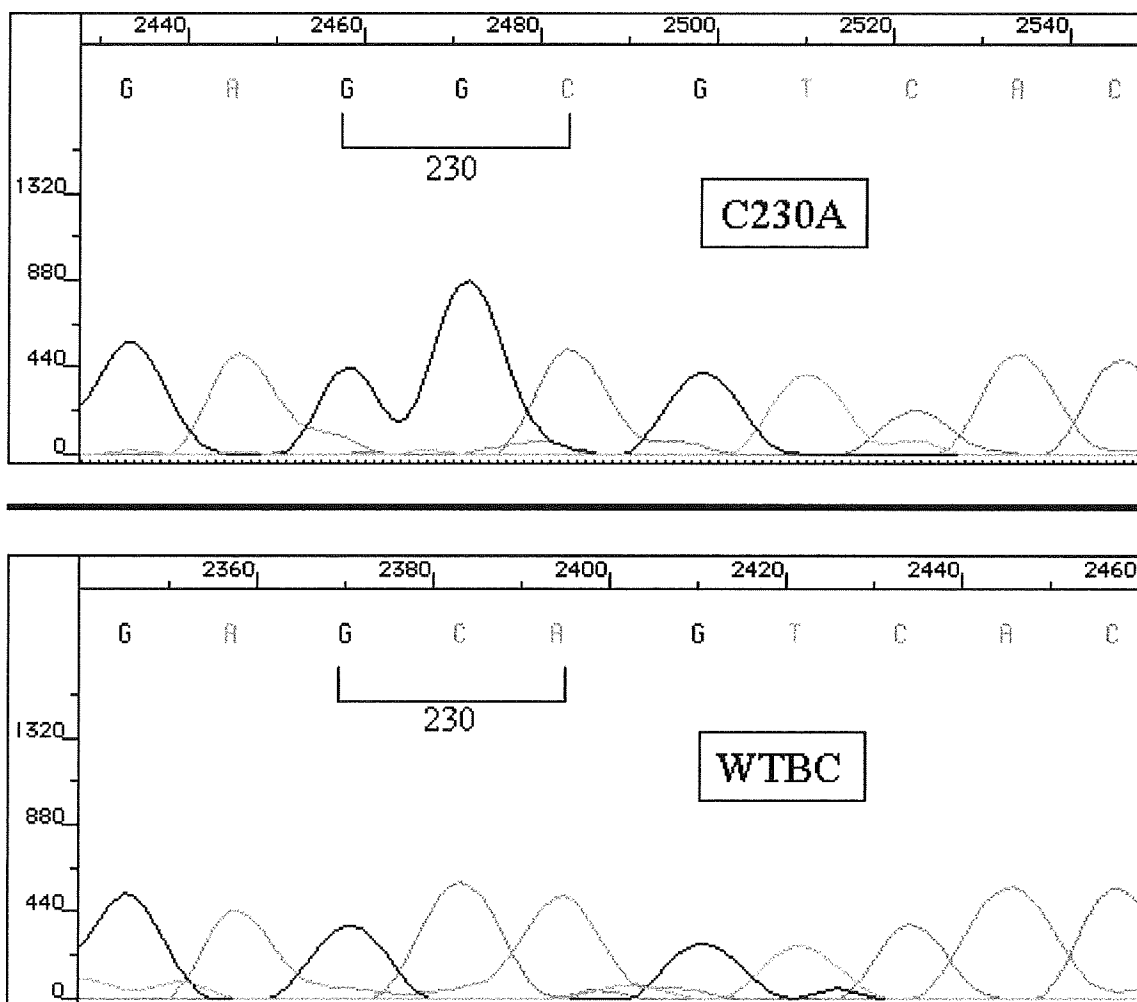


**Figure 4. Inactivation of lysine by NEM.**

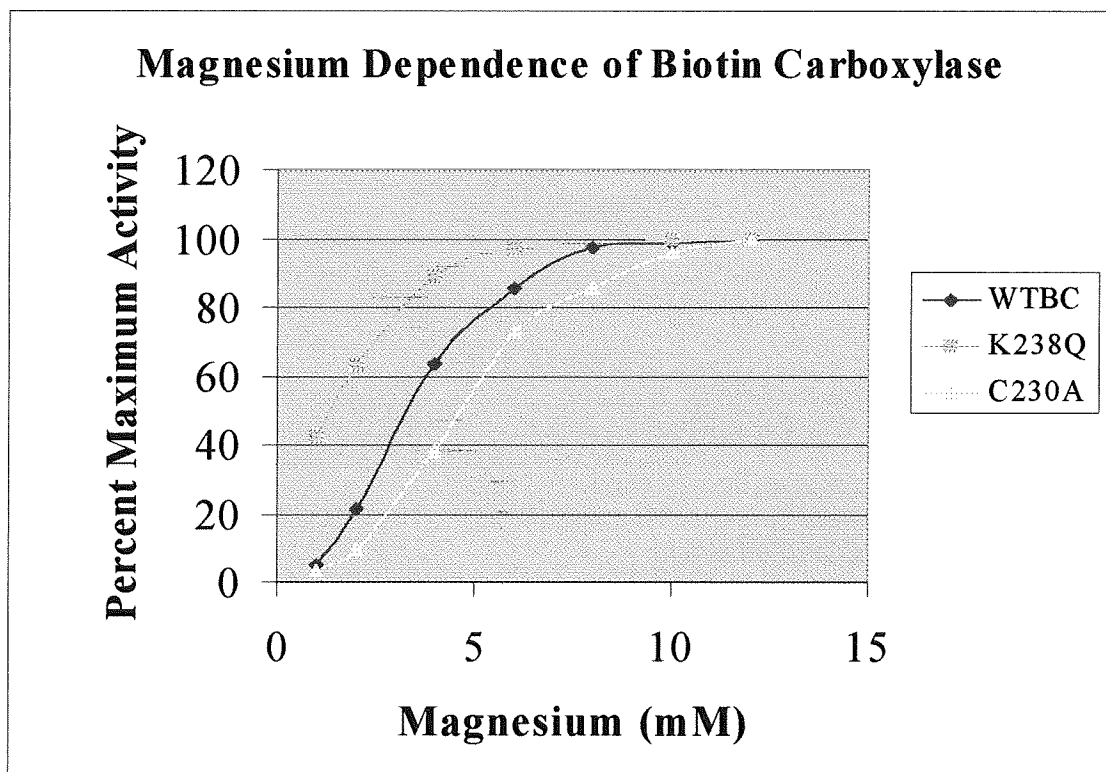


**Figure 5. Sequences for K238Q (Top) and WTBC (Bottom). The DNA coding for amino acid 238 is marked by brackets. The wild-type sequence (TTT) was changed to TTG in the K238Q mutant.**

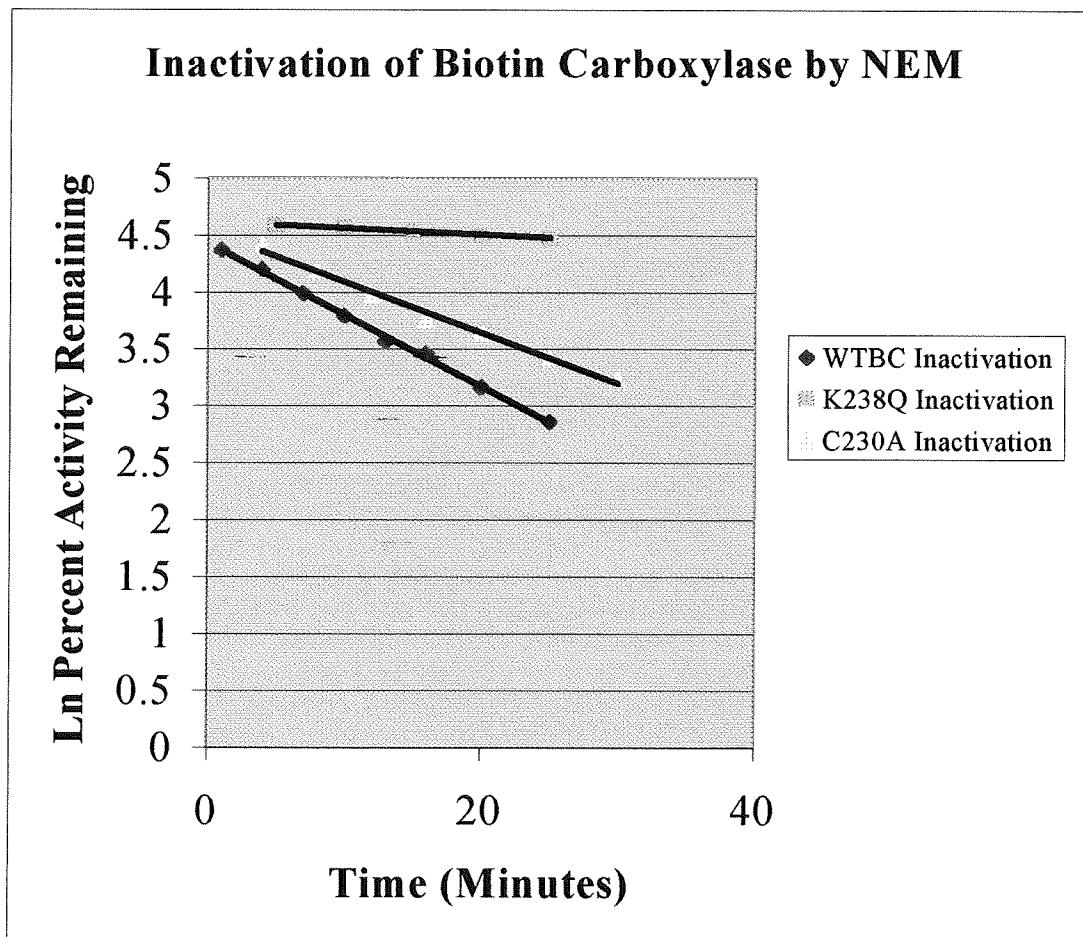




**Figure 6. Sequences for C230A (Top) and WTBC (Bottom). The DNA coding for amino acid 230 is marked by brackets. The wild-type sequence (GCA) was changed to GGC in the C230A mutant.**



**Figure 7. Magnesium Assay.**



**Figure 8. Inactivation of WTBC, K238Q, and C230A by NEM. Wild-type inactivated at a rate of 0.0632, K238Q at 0.0056, and C230A at 0.0448.**

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